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"INTERFERENCE" IN MIXED INFECTIONS OF BARTONELLA AND EPERYTHROZOON IN MICE *

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In the study of a bartonella which occurs naturally as a latent infection in the local "white-footed" or "deer" mouse, *Peromyscus leucopus novaboracensis,* it was found that this species of bartonella was regularly infective for splenectomized white mice. It was then soon noted that the appearance of *Eperythrozoon coccoides* in the blood of the infected mice was promptly followed by the complete disappearance of the bartonella as judged by stained blood films. This observation led to a number of experiments to test the effects of these two infections one upon the other.

Preparatory to the consideration of the experimental data, it may be well to make certain comparisons in regard to the infections in question. *E. coccoides* is of frequent occurrence in common white mice and is commonly latent in laboratory stock mice, its presence being demonstrable by splenectomy. Following the removal of the spleen, the organisms appear promptly (3 or 4 days) and usually in great numbers in the circulating blood, adhering to the surfaces of the erythrocytes or free in the plasma. The reticulocytes, which occur normally in limited number in mouse blood, are somewhat increased and early in the infection have great numbers of the eperythrozoa adherent to them, often

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grouped in a continuous layer extending for some distance in from the margins of the cells. The mature erythrocytes are, at this phase of the infection, relatively free of organisms but later on there is no longer any marked predilection for the reticulocytes. The morphology of the organism is by no means constant: on certain occasions coccoid forms predominate with but few rings; at other times the latter are present in great numbers. As the eperythrozoa diminish in number in the course of the infection, it may require some search to demonstrate true ring forms. While no exact blood studies have been undertaken, the notable increase in the reticulocytes suggests some degree of anemia. However, the infected animals are at no time obviously ill, nor is there pallor of the exposed skin of ears, feet and tail.

The infection produced in splenectomized white mice by the bartonella derived from the deer mouse is regularly severe and occasionally fatal. The organism appears in great numbers in the blood in from 3 to 8 days after inoculation and the infection soon reaches its height and subsequently runs a prolonged course characterized by remission and recrudescence. The initial rapid increase in bartonellae regularly terminates in a crisis characterized by great destruction of both red cells and microörganisms, the latter often not being microscopically demonstrable for a time. For several days following the crisis the animal is weak, somnolent, and there is staring of the fur together with extreme pallor of the exposed skin. However, no indication of hemoglobinuria has ever been observed. The fecal pellets are passed in long strings, owing to a great excess of intestinal mucus. The snipping of the tail to provide blood films is often followed by gangrene extending for a centimeter or more from the tip. Most deaths occur immediately following the onset of the initial crisis. Subsequent crises are in general less severe although death from bartonellosis has occurred 90 days after inoculation. Obviously death is due primarily to the sudden destruction of the red blood cells, the organs being pale as in an exsanguinated animal. The bartonellae at the height of infection occur in great numbers, some lying free but mostly distributed over the surface of the red cells. There is a sudden drop in body temperature at the onset of the blood crisis. Within 24 hours the rectal temperature may fall from 37.8°C. to below 32.5°C., the latter being the lowest reading that

could be obtained with the thermometer employed. Even with such very low temperatures recovery usually occurs, with readings gradually becoming higher so that, within a week of the onset, the temperature will have returned to normal.

It should be understood that *E. coccoides* occurs naturally in the common mouse, while the bartonella under consideration is derived from another host, the white-footed mouse. Many rodents carry latent bartonella infections, but none of the stock of white Swiss mice used has developed a bartonellosis on splenectomy.

EXPERIMENTAL

Bartonella infection was produced in each experimental animal by both subcutaneous and intraperitoneal injection of a suspension in citrated saline of the blood of either an infected *Peromyscus* or a common mouse. *E. coccoides* was always readily obtained from splenectomized stock mice and was similarly transmitted by the injection of the blood of an infected animal. The course of each infection was followed in the inoculated animal by the study at frequent intervals of blood films stained with Giemsa's stain and through the observation of such symptoms as occurred. Instruments employed in obtaining blood for films should always be sterilized between each operation. The bartonella of the deer mouse and *E. coccoides* are distinguished by their morphology and their distribution. The occurrence of rods and coccoids in chains and distributed quite generally over the surface of mature red cells has been regarded as satisfactory evidence of bartonellosis. The presence of rings in the plasma and on the margins of reticulocytes but never in chains has been considered sufficient for the diagnosis of eperythrozoon. Most forms of this organism are stained less intensely than the bartonella by Giemsa's method. While it would be quite impossible to identify individual coccoids, after gaining an acquaintance with morphology of the two organisms, no great difficulty is experienced in determining whether one or the other is present in appreciable numbers or both in mixed infection. The presence or absence of the two organisms at each observation, together with other data, is recorded in Table I.

Group I. Splenectomy was performed on each of 6 young white Swiss mice which, without preliminary examination of the

blood for eperythrozoa, were injected, 2 of them 11 days and one 10 days after splenectomy, with a suspension of blood of a deer mouse that had developed bartonellosis after splenectomy. Two of the splenectomized mice were injected with blood of a Peruvian mouse, *Phyllotis amico amico*. A blood examination several days later showed all 6 mice to be infected with *E. coccoides* irrespective of whether they received blood from *Peromyscus* or from *Phyllotis*. Hence there was reason to believe that all 6 mice of the series were infected with *E. coccoides* previous to splenectomy. Although all 6 were later again injected with mouse blood containing bartonellae in great numbers, no bartonellae appeared at any time in this group.

Group II. Splenectomy was performed on each of 11 white Swiss mice in batches on three successive days and, without preliminary blood examination, 2 to 4 days later they were injected with blood of the same deer mouse employed for group I. At the first observation, 4 days after inoculation, 5 showed bartonellae and 1 a mixed infection of eperythrozoon and bartonella. Nine days after inoculation the eperythrozoon had replaced the bartonella in this mouse, 1 had died of bartonellosis, and 9 were suffering in varying degrees from bartonellosis. On the following day eperythrozoa appeared in mixed infection in the bloods of 2 other mice and 6 days later the eperythrozoon had replaced the bartonella in all the surviving mice. As these animals were kept in two jars and were thrown together in the course of making blood films, the results suggested that one or more mice of this series were infected with *E. coccoides* before inoculation and that the infection spread gradually to all mice of the series.

Seventeen days after inoculation the 10 surviving mice were each treated by subcutaneous injection with 2.5 mg. of sulfarsphenamine, and in addition one half this amount was administered on the following day except to 1 mouse that died from the effects of the first injection. Sulfarsphenamine in adequate dosage has proved effective in eliminating both the eperythrozoon and the bartonella infections, and its injection in this instance resulted in the disappearance of the eperythrozoon from all the mice. Thirty-one days later the surviving mice were injected with blood from a mouse showing heavy bartonella infection. In 9 days 2 mice were dead of bartonellosis and 5 of the 7 survivors

were pale and extremely weak. Two (No. 9747 and No. 9751) showing the most severe symptoms were at this time injected with the blood of an eperythrozoon-infected mouse. In these the eperythrozoon promptly replaced the bartonella, but in 3 of the 5 other mice the bartonella also disappeared, to reappear long after in 1. Since it became apparent in this experiment that the bartonella could disappear spontaneously following the second crisis in a given animal, the conditions of this experiment were not suitable for demonstrating the effects of eperythrozoon injection on bartonellosis. The spread of eperythrozoon infection through the confinement of infected and noninfected mice in the same jar is suggested by the eventual appearance of this infection in mouse No. 9743, which was kept with the 2 eperythrozoon-inoculated mice.

Group III. Splenectomy was performed on each of 6 mice, and blood examinations made during the following month indicated that they were free of eperythrozoa. Three were then injected with blood containing *E. coccoides* and developed heavy infections. Fifteen days later all 6 were injected with *Peromyscus* blood showing great numbers of bartonellae. The 3 mice infected first with *E. coccoides* showed no bartonellae and presented a normal appearance over a period of 26 days, but on examination 56 days after the injection of bartonella, 2 were somewhat pale and presented a typical bartonellosis. This persisted for a period, but in both animals was again suppressed by a recurring eperythrozoon infection. At the height of the late-appearing bartonellosis, blood from each of the 3 eperythrozoon-treated mice, 2 showing at this time great numbers of bartonella, and 1 no organisms, was injected respectively into 3 mice, previously shown on splenectomy to be free of infection, in order to test for the presence of the eperythrozoon. Two of these developed eperythrozoon infection and 1 showed eperythrozoon and bartonella in mixed infection, the former organism soon replacing the latter.

The 3 untreated controls developed a severe bartonellosis and 12 days after inoculation were weak, pale, and showed staring of the fur, in marked contrast to the normal appearance of the mice inoculated with eperythrozoon 15 days prior to injection of the bartonella.

The results obtained in this group clearly show that eperythrozoon infection not only prevents the symptoms of bartonellosis but also prevents the multiplication of bartonellae in the blood. Under such circumstances, however, the bartonella may persist as a latent infection and later on it may gain the ascendancy when the eperythrozoon will then in turn become latent. Its presence, however, can be demonstrated by the injection of clean splenectomized mice. Thus a blood from which the eperythrozoon has long since disappeared microscopically may still be infective.

Group IV. For this series 7 mice were splenectomized and, having been proved to be free of eperythrozoa, 3 were injected with blood of an eperythrozoon-infected mouse. Ten days later these and the other 4 were injected with a suspension of pooled blood of 3 bartonella-infected mice. Bartonella was found on only one occasion in mixed infection with eperythrozoon in 1 of the eperythrozoon-infected mice, the observations having covered a period of 36 days from the inoculation of bartonella.

Group V. Eight recently weaned white Swiss mice were splenectomized, several on each of 3 consecutive days, and in from 2 to 4 days later were injected with heart's blood of mouse No. 9762, which had shown bartonellae 10 days previously but not when used for the present series. Since infection did not appear promptly in most of the inoculated mice, the entire group was reinoculated with heart's blood of mouse No. 9756, in which there were moderate numbers of bartonellae.

Ten days after the first inoculation all mice of the group were showing a heavy bartonella infection, and the first 4 of the group were inoculated with the blood of a mouse that had recently shown eperythrozoa, but this organism could not be demonstrated in a blood film taken at the time of inoculation. Since no eperythrozoon had appeared during the next 4 days, these 4 mice were again reinoculated with blood from a mouse showing moderate numbers of eperythrozoa. One animal, very weak from bartonellosis, died immediately. In the 3 eperythrozoon-treated mice, eperythrozoa appeared in considerable numbers 3 days after the last injection, but were greatly outnumbered by bartonellae. In the course of the following 5 days the bartonella was entirely replaced by the eperythrozoon.

The dosage of eperythrozoa injected less than 2 days previous to the crisis of bartonellosis was evidently too small to cause an immediate heavy infection and the symptoms were quite as severe in the eperythrozoon-treated mice as in the controls. On recovery from the crisis both treated and untreated mice became sleek and normally active and there was nothing distinctive in the general appearance of the two series. However, the continuing bartonellosis in the untreated mice was in each case associated with pronounced anemia, while the blood of the eperythrozoon-inoculated mice rapidly improved to approximately normal.

The ease with which eperythrozoon may be unintentionally introduced is illustrated in the results obtained in 3 mice not included in Table I, which were injected with blood showing great numbers of bartonellae. Prior to their inoculation 2 mice previously infected with eperythrozoon were injected with the same material, and the precaution was not taken of changing the hypodermic needle before injecting the clean group. Six days later, one of the 3 mice presented large numbers of eperythrozoa, and 2 a mixed infection of eperythrozoon and bartonella.

Discussion

While there are examples reported concerning the so-called "interference phenomenon" or "sparing effect" in regard to the influence of one infection upon another, the subject is one that appears to have received comparatively little attention. The results of the present study are of interest in that they are unusually clear-cut. The contrast between the appearance of groups of bartonella-infected mice at the time of the crisis of the infection and that of parallel groups infected with *E. coccoides* before being inoculated with the bartonella is very striking. While the former may appear so ill that recovery would seem improbable, the eperythrozoon-treated mice all maintain a normal appearance and remain sleek and normally active. The nature of the eperythrozoon and bartonella infections under consideration makes it possible to judge results on the basis not only of symptoms and pathological changes, but also on the course of the two specific infections in the blood as followed by periodic examinations of stained films. Observation is therefore more simple and

TABLE I

Observations Showing the Effects of Eperythrozoon coccoides on a Bartonellosis in Splenectomized Mice, as Based on the Incidence of the Two Organisms Found in Periodic Blood Examinations

Group	Mouse No.	Splenectomy	Eperythrozoon inoculation	Bartonella inoculation	Dates of observations: February 10	13	16	March 7	16	20	21	23	30	April 1	2	3	May 1	6
I	9723	Jan. 27	None	Feb. 6 and Mar. 16	E	E	E	E	E	E		E	E†					
	9724	" 26	"	Ibid.	E	E	E	E	E	E		E	O†					
	9725	" "	"	Ibid.	E	E	E	E	E	E		E	E†					
	9726	" "	"	Ibid.	E	E	E	E	E	E		E	E†					
	9727	" "	"	Mar. 26	E	O	E	E			E			E	s	s	O	O
	9728	" "	"	" "	E	E	E	E			E			E	s	s	O	O

Group	Mouse No.	Splenectomy	Eperythrozoon inoculation	Bartonella inoculation	Dates of observations: March 20	23	25	26	27	April 1	2	3	8	May 1	11	13	14	17	20	28	June 8	12	19	20
II	9743	Mar. 12	(Exposed*)	Mar. 16 and May 4	B		B	B		E	s	s	O	O	B	B		O	O	O	B	E†		
	9747	" 14	May 13	Ibid.	O		B			E	s	s	O	O	B	B		E	E	E	E	E†		
	9751	" 11	" "	Ibid.	EB	E	E	E		E	s	s	O	O	B	B		EB	E	E	O	E†		
	9740	" 12	None	Ibid.	O		B			E	s	s	O	O	B		B	O	O	O	O		O	†
	9741	" "	"	Ibid.	O		B			E	s	s	O	O	B		B	O	O	O	O		O	†
	9742	" "	"	Ibid.	O		B		EB	E	s	s	O	O	B		B	B	B	B	B		O	†
	9744	" "	"	Ibid.	B		‡																	
	9745	" "	"	Ibid.	B		B	B		E	s	‡												
	9746	" 13	"	Ibid.	O		B			E	s	s	O	O	B	‡								
	9749	" 14	"	Ibid.	B		B	B		E	s	s	O	O	O			B	B	O	B		O	†
	9750	" "	"	Ibid.	B		B	BE		E	s	s	O	O	B	‡								

Group	Mouse No.	Splenectomy	Eperythrozoon inoculation	Bartonella inoculation	Dates of observations																													
					March				April								May					June													
					4	11	21	30	4	8	15	17	18	22	29	1	2	3	4	6	4	5	10	12	19	20								
III	9730	Feb. 20	Mar. 26	Apr. 10	O	O	O		E		E	E	E	E		E				E			B	B	B	E	†						
	9731	" "	" "	" "	O	O	O		E		E	E	E	E		E				E			O	O	O	O	†						
	9733	" "	" "	" "	O	O	O		E		E	E	E	E		E				O			B	B	B	E	†						
	9735	" "	None	" "	O	O	O	O		O	B	B		B			B	B	B		B	†												
	9737	" "	"	" "		O	O	O		O	O	O	B	B			B	B			B		O		O	†								
	9739	" "	"	" "		O	O	O		O	B	B		B	B		B				B		O		O									

Group	Mouse No.	Splenectomy	Eperythrozoon inoculation	Bartonella inoculation	Dates of observations																													
					April	May										June					July													
					29	3	8	20	22	23	24	25	28	4	10	19	25	5	11	12	23													
IV	9758	Apr. 25	May 4	May 14	O	O	E	E	B	E		E	E	E	E	E	E	E			E†													
	9759	" "	" "	" "	O	O	E	E	E			E	E	E	E	E	E	E			E†													
	9763	" 29	" "	" "		O	E	E	E			E	E	E	E	E	E	E			E†													
	9756	" 25	None	" "	O	O		B	B			B	B	B	B	B	O	O	B	†													
	9757	" "	"	" "	O	O		B	B			B	B	B	B	O	B	O			O†													
	9761	" 29	"	" "		O		B	B	B	B	B	B	B	B	B	B	O			B†													
	9762	" "	"	" "		O		B	B			B	B	B	B	O	B	O†																

Group	Mouse No.	Splenectomy	Eperythrozoon inoculation	Bartonella inoculation	Dates of observations										
					July									August	
					5	10	11	15	18	19	22	24	27	1	
V	9781	July 1	July 15 and 19	July 5 and 12	O	B	B	B	B	B	BE	E	E	E†	
	9782	" "	Ibid.	Ibid.	O	O		B	B	B†					
	9783	" "	Ibid.	Ibid.	O	O		B	B	B	BEE(B)E			E†	
	9784	" 2	Ibid.	Ibid.	O	O		B	B	B	BEE(B)E			E†	
	9785	" "	None	Ibid.	O	O		B	B		B	B	B	B	
	9786	" 3	"	Ibid.	O	O		B	B		B	B	B	B	
	9787	" "	"	Ibid.	O	B	B	B	B		B	B	B	B	
	9788	" "	"	Ibid.	O	O		B	B		B	B	B	B	

E = eperythrozoon; B = bartonella; (B) = degenerating bartonella; O = no organism found; s = sulfarsphenamine injected.
* = kept in jar with mice No. 9747 and No. 9751; inoculated May 13 with eperythrozoon.
† = killed.
‡ = died of bartonellosis.
(In mixed infection precedence is given the preponderating organism.)

direct than in the case of virus diseases and most bacterial infections. The conditions are very similar to those which obtain in malarial infections, in which the organisms may be detected and their relative numbers estimated by microscopical examination of the blood. In fact, a similar effect in the tendency of one type of human malaria to exclude another has been repeatedly observed by malariologists.

The results obtained in malarial infections, however, do not appear to be as uniform as in the mixed bartonella-eperythrozoon infections. Thus James [1] (1931) found the incubation period of *Plasmodium falciparum* to be increased in a patient with *Plasmodium vivax* infection. On the other hand, both Antić [2] (1925) and Boyd and Kitchen [3] (1937) found that the simultaneous infection of patients with *P. vivax* and *P. falciparum* is frequently followed by the early appearance of both species, but that the latter soon gains the ascendancy while the former may entirely disappear for a long period. Later on *P. vivax* may reappear and replace the other species. There is difficulty in the interpretation of such cases on account of their limited number and of the possibility of spontaneous remissions such as occur when only a single infection is present. Although it would be possible to collect a considerable number of references to the literature in regard to the effects of one infection upon another, it does not seem appropriate to review the entire subject until more is learned of the principles basic to the phenomena under discussion.

In regard to terminology, while "interference," "antagonism," and "sparing effect" have been applied more generally to the visible effects of infections rather than to the actual presence and multiplication of organisms or viruses, it is doubtful whether it would be desirable to introduce a new term for the phenomena under discussion, in infections in which fluctuations in the number of organisms may be followed by periodic examination of stained films. The term "interference" appears to be widely used and quite generally applicable.

In a previous paper (Tyzzer and Weinman,[4] 1939) it was noted that with the great variety of blood infections found in the vole, *Microtus pennsylvanicus pennsylvanicus*, no mixed infections of bartonella and eperythrozoon were found. A series of splenectomized white mice was experimentally infected with the

vole bartonella but certain failures subsequently noted in the inoculated mice have since been found to have occurred in individuals that were carrying *E. coccoides* infection.

Preliminary experiments have shown that it is possible in some instances to infect splenectomized voles with *E. coccoides* of the common mouse. Two of four old voles inoculated showed infection, which in one was light and transient, in the other quite heavy and prolonged. On being injected 15 days later with *Haemobartonella microti,* both developed a well marked bartonellosis. Thus *E. coccoides* in an alien host has failed to interfere with the development of a bartonella occurring naturally in that host.

No serological basis is known for the protective action of preëxistent *E. coccoides* infection against the *Peromyscus* bartonella in the tame white mouse, nor for the suppressive action of the former when introduced in established infections of the latter. The uncomplicated bartonellosis continues for many weeks with an abundance of organisms in the blood, so that the sudden microscopic disappearance of the bartonella attending the increase in the eperythrozoa is difficult to explain. The presence in the blood of considerable numbers of eperythrozoa is evidently essential in order to replace the bartonellae. Furthermore, a bartonellosis with great numbers of organisms in the blood may develop after the eperythrozoon infection has become latent, though still demonstrable by transfer to clean splenectomized mice. Long-standing bartonella infection affords no protection against eperythrozoon infection, as shown by a number of test animals. Such animals cannot be reinfected, however, by reinoculation with bartonella.

While elevation of body temperature is regarded as important in the treatment of syphilis involving the central nervous system by induced malaria, such is not the case in the suppression of the bartonella by the eperythrozoon. The temperatures taken in eperythrozoon-treated and untreated mice with bartonellosis from before the crisis up to their symptomatic recovery averaged slightly lower in the treated mice even during the period of the disappearance of the bartonella.

It is quite possible that "chemotherapeutic interference," as reviewed by Findlay [5] (1939), may in some way be related to

the type of interference in which one infection prevents or replaces another.

While the existence of eperythrozoon or of bartonella infection in experimental animals may be ascertained by splenectomy, it is important to observe certain precautions in order to prevent accidental transmission when employing groups of animals which are confined in a single cage or jar. Thus the snipping of the tails of mice or rats in the preparation of blood films may lead to the smearing of the surroundings and often of the fur or skin of another animal, which may then lick off the infective material. Carelessness in regard to the sterilization of the hypodermic needle when inoculating series of animals, or of instruments used in obtaining blood for films, or in punching or otherwise marking the ears, may result in accidental infection.

The practical bearing of the phenomena under discussion lies in the potentiality of unrecognized infections on occasion to reverse experimental results. An interesting field is opened in testing the possibilities of effecting protection against serious infections by vaccinating with harmless ones.

Summary

A bartonella occurring naturally in the local "white-footed" mouse *Peromyscus leucopus novaboracensis* is pathogenic for splenectomized normal white mice, producing a severe and occasionally fatal anemia.

E. coccoides infection occurring naturally or induced in splenectomized white mice prior to their inoculation with the *Peromyscus* bartonella interferes with the development of the latter and prevents all symptoms. In such animals the bartonella, although not microscopically demonstrable, persists latently and may, after many weeks, replace the eperythrozoon infection which in turn becomes latent.

E. coccoides, introduced after the bartonellosis is established, promptly suppresses the latter infection so that bartonellae are no longer demonstrable in stained blood films.

The eperythrozoon of the white mouse has developed in a certain proportion of the splenectomized voles (*Microtus pennsylvanicus pennsylvanicus*) inoculated, but in this foreign host does

not interfere with the development of severe bartonellosis due to the vole bartonella.

The possibility of the reversal of experimental results through the presence of unrecognized infection is pointed out, and that there may be a possible utilization of the principles of interference in the field of vaccination is suggested.*

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* Subsequent to submitting this paper for publication, additional observations were made on eperythrozoon and bartonella infections occurring naturally in the gray-backed deer mouse, *P. maculatus gracilis*. An eperythrozoon infection, becoming apparent in two animals after splenectomy, kept in suppression a bartonella infection for a period of about 6 weeks, at the end of which the latter organism gained the ascendancy in both animals. Thus "interference" is effective following splenectomy in eperythrozoon and bartonella infections coexisting in their natural host.

EXPERIMENTAL OCULAR TUBERCULOSIS IN NORMAL AND IMMUNIZED RABBITS *

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The experimental work on ocular tuberculosis has been so completely reviewed by both Finnoff [1] and Woods [2] that only studies pertaining directly to our problem will be discussed.

Kostenitsch and Wolkow [3] in 1893 described the development of ocular tuberculosis in the rabbit following an intravenous injection of avian tubercle bacilli. Friedrich and Nösske [4] in 1899, Stock [5] in 1903, and Daels [6] in 1907 have reported the production of ocular tuberculosis in a fairly high percentage of rabbits following intravenous injections of large numbers of tubercle bacilli. The intravenous method, however, has been replaced for the most part by intracarotid injection. Lagrange [7] in 1898 was the first to report the localization of tubercles in the eye after the injection of tubercle bacilli into the carotid artery. The same method was used by Finnoff,[8] who injected killed tubercle bacilli by this route and produced tubercles in the choroid. We have been able to confirm his results in this laboratory. Our experiments, like those of Finnoff, indicate that the organisms do not localize in the eye if they are injected in the form of a fine suspension. In fact, a very coarse suspension is necessary in order to produce ocular tuberculosis by this method. Insufficient attention seems to have been given to this finding.

There are numerous reports on the effect of sensitization on the development of ocular tuberculosis. The studies of Lagrange,[9] of Samojloff,[10] and of Woods, Burky and Friedenwald [11] should be mentioned. These observers have described the effect of general sensitization on the intra-ocular reactions to either tubercle bacilli or tuberculin. As far as we have been able to ascertain,

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there are no reported studies on the effect of sensitization upon the development of ocular tuberculosis produced by intravenous injection of relatively small doses of living tubercle bacilli.

E. L. Opie and his associates, to whom we are indebted for this material, have for many years been studying experimental tuberculosis in rabbits with special emphasis on the relationship of sensitization to immunization. As complete autopsies were performed on all control or immunized rabbits that died or were killed during the course of these experiments, it seemed probable that a careful anatomical study of the eyes might give some insight into the pathogenesis of experimental ocular tuberculosis. An opportunity would also be afforded to compare the development and progress of ocular tuberculosis in normal and in immunized rabbits and its relationship to systemic tuberculosis and especially to tuberculosis of the lungs.

Method

The preparation of the vaccine, the method of immunization and infection of the animals have been described in detail by Opie and Freund.[12]

The infecting dose was a suspension of tubercle bacilli diluted so that 0.5 cc. contained 0.00001 mg. The content of each tube was stirred vigorously in order to break up, as far as possible, any clumps which might remain. The material was injected into a marginal ear vein. Amounts of suspension equivalent to 0.00001 and 0.000001 mg. were cultured on slants of Petragnani's media. There was considerable variation in the number of viable organisms; however, in the majority of instances the number of colonies obtained from 0.00001 mg. was between 40 and 200.

Experimental

Most of the experiments were on the protective effect of the intradermal inoculation of rabbits with heat-killed bovine (Ravenel) or human (Jamaica 22) tubercle bacilli against subsequent infection with a small dose (0.00001 mg.) of virulent bovine tubercle bacilli (Ravenel strain). A few animals were infected with a larger dose (0.01 mg.) of the same strain (Table I). The disease produced was chronic, few animals dying within less than 3 months. Animals were not killed unless *in extremis* until 2 years from the date of infection, at which time the experiments were concluded. Both human and avian tubercle bacilli produced tuberculosis in the eyes of a smaller number of rabbits.

Since the ocular tuberculosis will be described in considerable detail, other findings will be briefly summarized. The chief difference between the immunized and infected control rabbits was that in the former group some animals survived infection, and the average duration of life was greater. No consistent differences were noted in the extent or character of the tuberculosis in the two groups. Usually, the most extensive involvement was in the lungs; however, lesions of other viscera, especially appendix, ileocecal junction and kidneys, were often conspicuous. Involvement of the spleen and liver was infrequent. Pulmonary cavitation was not unusual, and lesions with some calcification were often found. Histologically, the tuberculous lesions were similar to those in humans save for the relative scarcity of giant cells. There was often extensive amyloidosis which occurred chiefly in the spleen, kidneys and liver.

The eyes, removed from the animals at autopsy, were placed in formalin. They were opened by a median incision, and after removal of the vitreous humour and lens, were examined for gross evidence of tuberculosis, the size and anatomical location of any tubercles being noted. Routine histological sections were prepared from most eyes, and all lesions about which there was any doubt were studied microscopically. When these examinations were completed, the ocular tuberculosis was studied in relation to the time of development, duration of life, amount of pulmonary tuberculosis, degree of sensitization and the presence of complement-fixing antibodies.

Results of Experiments

The number of rabbits, the method of immunization, the amount of the infecting dose, the average duration of life and the number of animals developing ocular tuberculosis are shown in Table I.

From this table it is evident that the immunized animals in the groups receiving an infecting dose of 0.00001 mg. lived, on the average, longer than did the infected controls. The incidence of tuberculosis of the eye was also considerably less. In the four groups of immunized animals the incidence of ocular tuberculosis varied from 10 to 36.7 per cent, and was 27.2 per cent for this

entire group, whereas it was 49.1 per cent in the nonimmunized rabbits. It is of interest that the difference in the incidence of ocular tuberculosis between the infected controls and immunized rabbits is about 2.96 times its standard deviation.

TABLE I

The Incidence of Ocular Tuberculosis in Normal and Immunized Rabbits Infected Intravenously with Bovine Tubercle Bacilli (Ravenel)

Groups	Infecting dose (mg.)	Number of rabbits	Ocular tuberculosis		Average length of life (days)
			Number	Per cent	
Infected controls	0.00001	57	28	49.1	188.7
Treated with heat-killed Ravenel (4 injections)	0.00001	43	14	32.6	313.4
" " heat-killed Jamaica 22. (4 injections)	0.00001	20	2	10.0	278.4
" " heat-killed Jamaica 22. (1 injection)	0.00001	43	10	23.3	211.6
Treated in various ways	0.00001	30	11	36.7	337.6
Infected controls	0.01	12	10	83.4	88.0
Treated with heat-killed Ravenel (6 injections)	0.01	14	8	57.1	87.7

When a larger infecting dose (.01 mg.) of tubercle bacilli was injected intravenously, the incidence of tuberculosis of the eye was greater and the average length of life considerably less. Ocular tuberculosis developed in 10 of 12 infected control rabbits and in 8 of 14 of those immunized and subsequently infected. This small group of animals will not be included in the tables or observations that follow.

Time of Development of Ocular Tuberculosis. In 18 of 28 infected control rabbits that developed ocular tuberculosis, the lesion appeared sooner than 164 days following infection, whereas it was found within this period of time in only 4 of 37 immunized rabbits (Table II). Moreover, the earliest example of ocular tuberculosis found at autopsy in the group of infected controls was at 93 days after infection, and in the immunized group at 157 days. These observations show that there is a considerable delay in the development of ocular tuberculosis, especially in the immunized group. In many instances the tubercles found in the eye were apparently recent, and it is probable that the tuberculosis in the eye was a secondary development rather than a direct

result of the original intravenous injection. Some studies made by Deane C. Hartman in this laboratory are of interest in this connection and tend to support this observation. He made repeated ophthalmoscopic examinations of the eyes of an additional large group of infected rabbits for a period of more than 1 year following infection. In no instance did he observe tuberculosis

TABLE II
The Time of Development of Ocular Tuberculosis in Infected Controls and Immunized Rabbits

Groups	No. of rabbits	No. developing ocular tuberculosis	
		Within 164 days	After 164 days
Infected controls	28	18	10
Immunized rabbits	37	4	33

in the eyes of these rabbits sooner than 5 months after infection. If it were the result of the original injection, it would probably have developed before this time.

Relationship to Pulmonary Tuberculosis. At the time of autopsy the amount of tuberculosis in the lungs of all the rabbits was recorded in percentage of the cut surface of the lungs occupied by tuberculous lesions. It seemed desirable to determine whether the ocular tuberculosis bore any relationship to the amount of pulmonary tuberculosis. The animals were accordingly separated into two groups, depending upon the extent of

TABLE III
Incidence of Tuberculosis of the Eyes of Rabbits in Relation to the Extent of Lung Involvement

Groups	Per cent of lung involvement	No. of rabbits	With ocular tuberculosis	
			Number	Per cent
Infected controls	50% or less	36	13	36.1
	More than 50%	21	15	71.4
Immunized animals	50% or less	80	16	20.0
	More than 50%	50	21	42.0

lung involvement. The results are shown in Table III. It is evident that the incidence of ocular tuberculosis in both the infected controls and immunized rabbits is considerably smaller in animals in which less than 50 per cent of the lung is involved than in rabbits in which more than 50 per cent is involved. When the

control and immunized rabbits are considered together, ocular tuberculosis is found in 29 of 116, or 25.0 per cent, of those with less than half of the lung tissue involved, and in 36 of 71, or 50.7 per cent with more than half involved. In other words, the ocular tuberculosis occurred in direct proportion to the amount of tuberculosis in the lungs. However, in some instances it developed in the eyes when there was relatively scant pulmonary tuberculosis.

Relationship to Sensitization. Tuberculosis of the eye was next considered in relation to the amount of skin sensitivity to tuberculin. In the infected control group we have considered the highest tuberculin reading subsequent to infection as the index of sensitivity for each animal although this may have changed considerably just prior to death. For the immunized group, the tuberculin test just prior to infection has been used. On this basis the animals with ocular tuberculosis and those without were considered in relation to the degree of tuberculin skin sensitiveness. So far as we were able to determine, the degree of skin sensitivity did not bear any relationship to the occurrence of ocular tuberculosis.

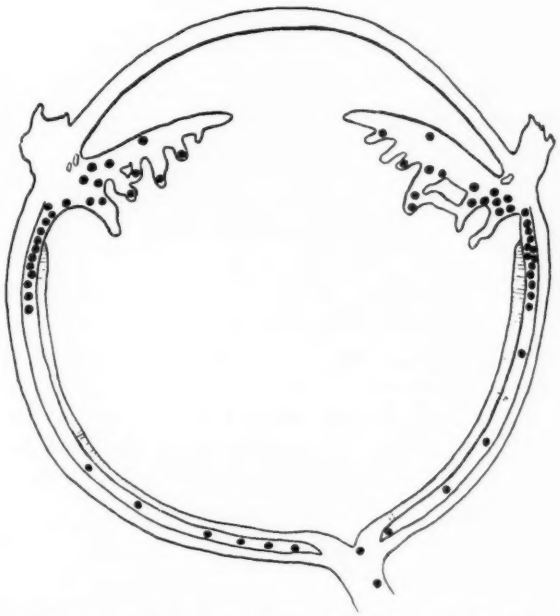
Complement Fixation. Complement fixation tests were done on all the animals, but no relationship was found between antibody formation and the development of ocular tuberculosis.

Pathological Lesions

In a few instances tubercles were observed in the iris and anterior chamber of the eyes during life (Figs. 1–5). The location and size of the tubercles were noted at autopsy and the location is shown diagrammatically in Text-Figure 1. Tubercles were found to occur most frequently in the posterior part of the iris and in the region of the ciliary body. When the eyes were extensively involved, the tubercles in the ciliary body were frequently large and caseous and perforated the eye at the corneoscleral junction. The tubercles in the choroid were readily seen through the retina and measured from pin point size up to about 7 mm. in diameter. They occurred in the ciliary processes frequently enough to suggest that these processes may be of significance in causing bacteria to localize in the eye. The significance of this relation has been pointed out in an earlier publication.[13]

Tubercles in smaller numbers were present in the anterior part of the choroid and in two instances involved the optic nerve.

The microscopic sections of eyes with tuberculosis from the infected controls were compared with those from the immunized rabbits with special reference to the size of the tubercles, type of cellular reaction, amount of fibrous connective tissue, degree



Text-Figure 1. Distribution of ocular tubercles as found at autopsy.

of caseation, number of giant cells and increase in pigmented cells. No significant difference was noted in the type of inflammatory reaction. The microscopic findings apparently depended largely upon the age and size of the tubercles. In a considerable number there were many polymorphonuclear leukocytes. Epithelioid cells and lymphocytes were present in practically every instance. There was a considerable amount of fibrous connective tissue in the tubercles of about one third of the eyes. There were varying degrees of caseation. Frequently, large numbers of pigmented chromatophores were present in the tubercles, especially

in those of the choroid. Giant cells were relatively rare and were observed in only three of the eyes sectioned.

Sections stained by the Ziehl-Neelsen method were prepared in many instances and large numbers of acid-fast bacilli were found in the tubercles in all the eyes examined in this manner. Examples of the various types of tuberculosis observed are illustrated by photomicrographs (Figs. 6–11).

Summary and Conclusions

1. Tuberculosis developed in the eyes of 28 (49.1 per cent) of 57 infected control rabbits injected intravenously with 0.00001 mg. of bovine tubercle bacilli.

2. Tuberculosis developed in the eyes of 37 (27.2 per cent) of 136 rabbits immunized in various ways with either heat-killed human or bovine tubercle bacilli and subsequently infected with 0.00001 mg. of bovine tubercle bacilli.

3. Larger amounts (0.01 mg.) of bovine tubercle bacilli produced ocular tuberculosis in 10 (83.4 per cent) of 12 infected controls as contrasted with 8 (57.1 per cent) of 14 immunized rabbits.

4. Tuberculosis was not observed in the eyes of rabbits infected with 0.00001 mg. of bovine tubercle bacilli sooner than 3 months after infection. It appeared considerably earlier in the infected controls than in the immunized animals.

5. The incidence of ocular tuberculosis was closely related to the amount of tuberculosis in the lungs.

6. Tubercles were observed most often in the anterior segment of the eyes and occurred relatively frequently in both the ciliary body and ciliary processes.

7. Skin sensitivity to tuberculin did not seem to have any influence on either the gross or microscopic characteristics of tuberculosis in the eyes.

8. These experiments indicate that tuberculosis develops frequently in the eyes of infected rabbits. Immunization preceding infection affords some protection against the occurrence of ocular tuberculosis.

Note: We wish to thank Jules Freund and R. W. Linton for their help in accumulating this material.

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Description of Plates

Plate 32

Figs. 1 and 2. Rabbit No. 1. Tuberculosis of the iris. Both eyes have been fixed in 10 per cent formalin and the corneas removed. This rabbit had been immunized with four intradermal injections of 0.025 mg. of heat-killed bovine tubercle bacilli (Ravenel) and infected with 0.00001 mg. of bovine tubercle bacilli. The rabbit died 383 days after it was infected.

Fig. 3. Rabbit No. 2. A tubercle in the anterior chamber near the iris. This rabbit was treated in the same manner as rabbit No. 1. The photograph was taken approximately 22 months after infection. The highlights have been removed.

Figs. 4 and 5. Rabbit No. 3. Extensive tuberculosis of both eyes in an infected control that received 0.01 mg. of human (Jamaica 22) tubercle bacilli intravenously. Photograph was taken about one year later. *Right eye:* There is extensive clouding of the cornea, with extensive vascularization at the corneoscleral junction, most marked on the lateral aspect. *Left eye:* There is considerable clouding of the cornea, as well as several large tubercles in the anterior chamber. A highlight in the photograph has been obliterated to avoid confusion wtih the tubercles.

FIG. 1

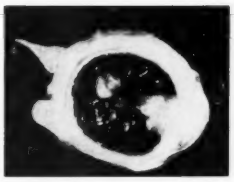

FIG. 2

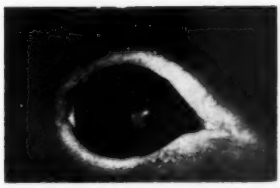

FIG. 3

FIG. 4

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FIG. 5

Experimental Ocular Tuberculosis

PLATE 33

FIG. 6. Rabbit No. 4. A large tubercle involving most of the ciliary body and part of the iris. This rabbit died from tuberculosis 164 days after an intravenous injection of 0.00001 mg. of bovine tubercle bacilli. × 13.

FIG. 7. Rabbit No. 5. Tubercles in the ciliary processes on the posterior part of the iris. This rabbit died from tuberculosis 160 days after receiving an intravenous injection of 0.00001 mg. of bovine tubercle bacilli. × 100.

6

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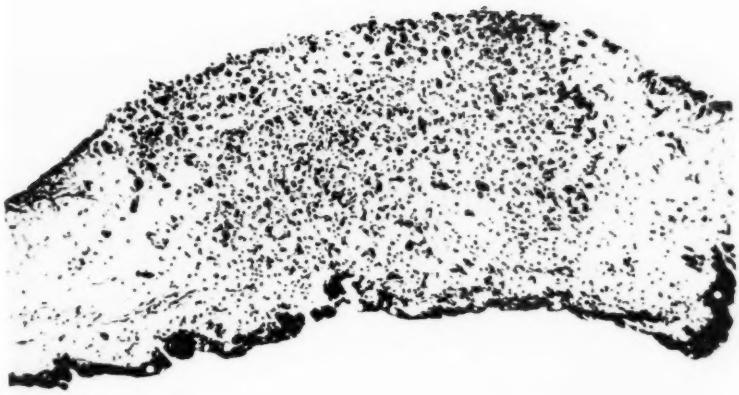
PLATE 34

FIG. 8. Rabbit No. 6. A tubercle in the iris composed largely of epithelioid cells, lymphocytes and pigmented cells. This rabbit died from tuberculosis 180 days following an intravenous injection of 0.00001 mg. of bovine tubercle bacilli. × 100.

FIG. 9. Rabbit No. 7. Several small localized tubercles in the iris composed largely of mononuclear leukocytes. This rabbit had been immunized with one injection of killed human tubercle bacilli and subsequently infected with an intravenous injection of 0.00001 mg. bovine tubercle bacilli. The animal died 230 days following infection. × 180.



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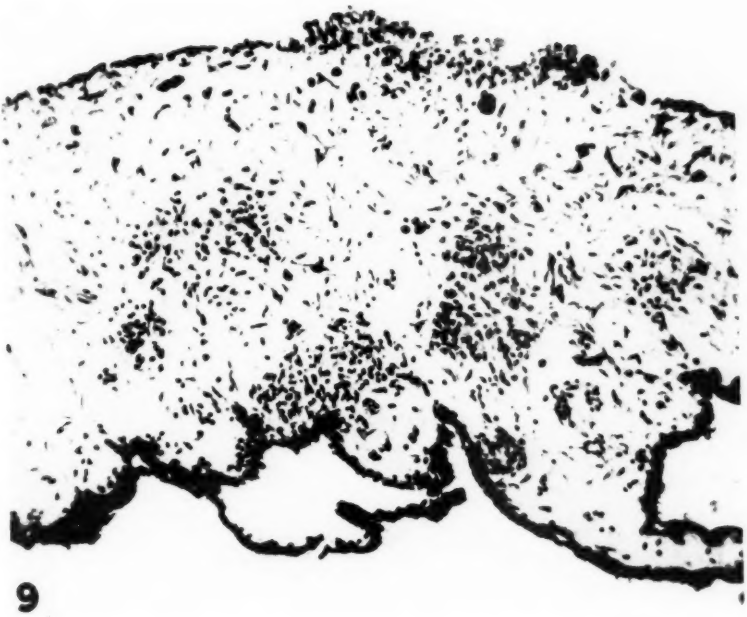

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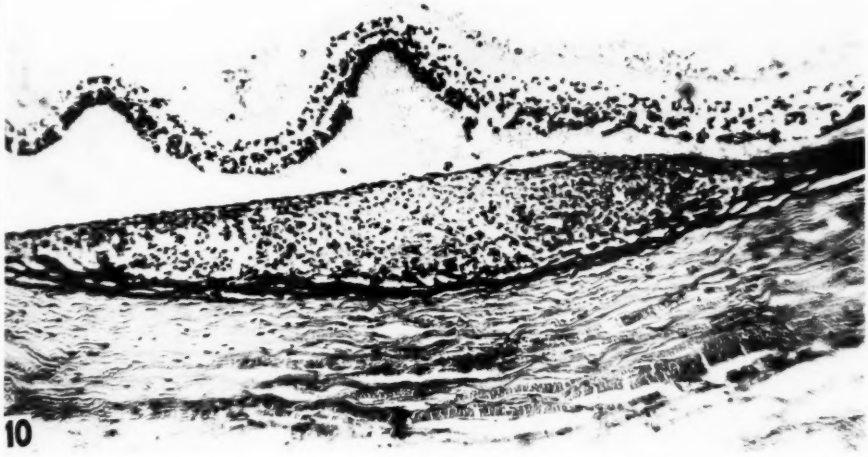
PLATE 35

FIG. 10. Rabbit No. 8. A tubercle in the choroid. Note the large number of pigment-containing cells and also evidence of edema beneath the retina. This rabbit died from tuberculosis 156 days after an intravenous injection of 0.00001 mg. of bovine tubercle bacilli. × 90.

FIG. 11. Rabbit No. 9. Several large tubercles with beginning caseation surrounding the optic nerve. The nerve tissue is extensively infiltrated with lymphocytes. The rabbit was treated in the same manner as rabbit No. 7 and died 214 days after infection. × 15.



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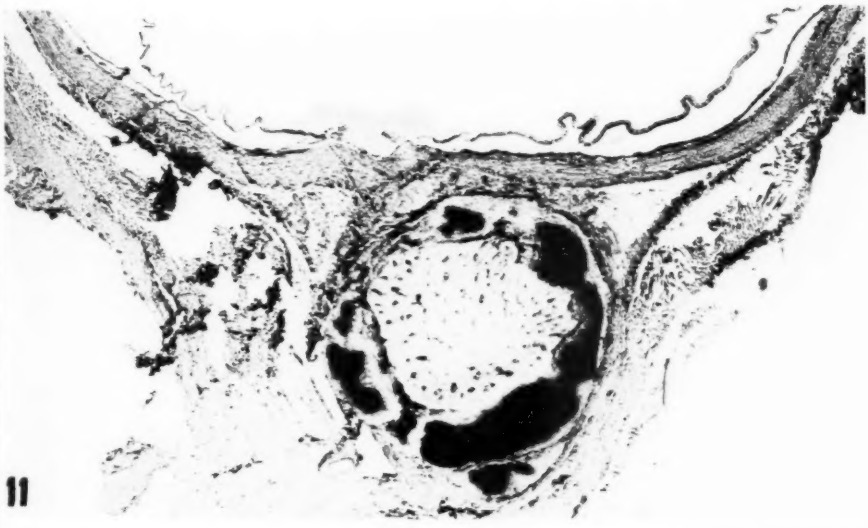

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Angevine and Huntington Experimental Ocular Tuberculosis

EXPERIMENTAL MEASLES *

The Lymphoid Tissues of Animals Inoculated with the Virus of Human Measles

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The prodromal stage of measles in man is accompanied by a specific giant cell reaction in lymphoid tissues. The present study was planned to utilize this reaction as a criterion of the transmissibility of measles from man to animals.

Literature

Numerous attempts have been made to produce measles in animals. Conflicting results were obtained when rabbits and guinea pigs were used as subjects and clinical and hematologic data were made the criteria of success or failure. Successful transmission of measles from man to rabbits, based on clinical manifestations, has been claimed by several investigators.[1-4]

Nevin and Bittman [1] injected rabbits intramuscularly with blood obtained from patients with measles in the early eruptive stage. The rabbits developed signs of measles lasting from 5 to 14 days. Control rabbits receiving blood from healthy donors or from patients with diseases other than measles did not develop similar clinical signs. Nevin and Bittman passed the virus serially through five rabbits and produced clinical signs of measles in a monkey injected with blood drawn from the fifth rabbit.

Several workers,[5-10] on the other hand, have found rabbits immune to measles. Purdy [6] showed that leukopenia and temperature variations similar to those considered significant by Scott and Simon,[4] may occur in normal rabbits. Enders [10] suggested that the "positive" results reported by a number of Japanese investigators may have been due to accidental infection by a virus other than that of measles. This explanation appears

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the more likely in view of the fact that some of these authors reported transmission of measles to mammals and birds of several species generally regarded as resistant.

Reports as to the transmissibility of measles from man to guinea pigs are equally conflicting. Duval and D'Aunoy [3] noted leukopenia, fever, hemorrhagic nephritis and inconstant coryza in their animals. They found that the virulence of the virus was increased by serial animal passage. Some of the animals inoculated with the more potent virus died, while others recovered and were immune to subsequent inoculation. Enders [10] stated that Ueda and Kasahara [11] also produced systemic changes in guinea pigs injected with blood removed from patients with measles. They found an interstitial pneumonia, with perivascular infiltrations of large "mononuclear cells," in their animals and produced clinical signs of measles in monkeys injected with emulsions of the lungs and testes of infected guinea pigs. Enders was unable to confirm these results and found similar pneumonic lesions in control guinea pigs. Negative results with guinea pigs have been reported also by Kraft,[9] Nicolle and Conseil [12] and Blake and Trask.[13]

It is more generally agreed that monkeys are susceptible, at least in a mild form. Blake and Trask [13] and Blake, Trask, Culotta and Beebe [14] injected monkeys intratracheally with nasopharyngeal washings obtained from patients with measles. Their animals, after a short period of incubation, developed an enanthem, malaise, conjunctivitis and eruption. The lesions found in the skin and mucous membranes showed histologic changes similar to those described by Mallory and Medlar [15] in Koplik's spots and in the cutaneous lesions of measles in man. The virus could be maintained for a limited period by animal passage. Successful inoculation of monkeys has been reported by others.[1,6,10,16] Rake and Shaffer [16] propagated the virus of measles on the chorio-allantois of chick embryos and infected monkeys with the virus grown in this manner. They were able to recover the virus from monkeys thus infected and propagate it again by incubation in fertilized eggs.

A few investigators [17,18,19] have found monkeys resistant to inoculation with blood or nasal secretions obtained from patients with measles. Sellards and Wentworth [18] injected five monkeys

intraperitoneally with blood. Two of the monkeys developed leukopenia and one of these a slight rash. Blood taken from the latter at the onset of the leukopenia and 5 days before the appearance of the rash failed to cause measles when injected into an adult human volunteer. This is in contrast to the results obtained by Nicolle and Conseil [12] who reported the production of measles in a child injected with blood taken from a monkey which had been infected with measles experimentally. Degkwitz[20] and Degkwitz and Mayer [21] produced definite clinical signs of measles in only 10 per cent of their monkeys and Kraft [9] had equally disappointing results.

In summary it may be said that a majority of observers agree that monkeys are susceptible to measles. Opinion as to the susceptibility of animals below the order of primates is more sharply divided. A majority of the authors cited have found guinea pigs and rabbits to be immune.

Much of the uncertainty indicated in these reports could have been dispelled had search been made for the histologic lesions described independently by Warthin [22] and by Finkeldey [23] in 1931. This omission is hardly surprising, however, in view of the fact that a description of these lesions is omitted in recent editions of three widely used textbooks of pathology,[24,25,26] while a fourth [27] mentions them only briefly. The diagnostic significance of the Warthin-Finkeldey giant cell reaction has been confirmed on many occasions.[28-32] Its presence may be accepted as a sign of impending measles and it probably does not occur in any other infection. This statement is made despite the recent report of Tomlinson [33] who found similar cells in tonsils removed from a child who subsequently developed chickenpox. It is probable that this patient had measles in such a mild form as to be unrecognized, the chickenpox developing as an intercurrent infection.

The Warthin-Finkeldey giant cell is a large, multinucleate, round or irregular syncytial cell. It has a variable number of lightly staining nuclei characteristically arranged in a grapelike cluster near the center of the cell.[22] In the tonsil these cells are most numerous beneath the epithelium of the crypts and that of the surface, but also occur within the germinal centers of the lymphoid follicles. Warthin suggested that they arise by "amitotic division in hyperchromatic cells resembling lymphocytes in

the subepithelial layer and in the germinal centers." He looked upon these cells as a defensive mechanism and considered it likely that they contain the infectious agent of measles. Finkeldey [23] suggested that the reaction may be in the nature of an allergic response. Cells of a similar nature and significance have been found in the appendix.[28-32] Hathaway [34] found them in the spleen and lymph nodes of a child who died of peritonitis during the prodromal stage of measles. Multinucleate giant cells containing cytoplasmic inclusion bodies have been found in the respiratory tract during the prodromal stage of measles [35] and Masugi and Minami [36] reported the presence of giant cells in the epithelium of the respiratory tract 4 days after the development of the rash of measles. Stryker [37] recently questioned the specificity of the giant cells described by Masugi and Minami. He found four distinct types of giant cells in the lungs and respiratory tract, but considered only one of these specific for measles.

With some of these reports in mind, it was decided to inoculate a number of animals with blood taken from patients with measles and examine their organs for the presence of Warthin-Finkeldey giant cells.

Experimental

Material and Methods. The following experiments were performed at intervals from 1938 to 1940, whenever the necessary clinical material became available. In all, 5 patients were used. These were negative, both clinically and serologically, for syphilis. All had unmistakable signs of measles, the first 3 in a severe, the last 2 in a mild form. The diagnosis, in each instance, was substantiated by an experienced clinician. Patients Nos. 1, 2 and 3 were children; Nos. 4 and 5 young adults. Blood was taken from them within 24 hours after the appearance of the rash and "citrated." A portion was cultured aerobically and anaerobically on blood agar and in Douglas broth. There was no growth in any of the cultures. The remainder of the blood was injected immediately into rabbits, white rats, guinea pigs and monkeys, as follows: On April 12, 1938, 4 rabbits were injected intravenously with blood obtained from patient No. 1 and on April 15, 4 with blood removed from patient No. 2. One rabbit in the second group received 4 cc., the other 7 rabbits 2.5 cc. each. On April

18, blood was taken from patient No. 3 and injected into 1 rabbit, 5 white rats and 3 guinea pigs. The rabbit received 6 cc. intravenously, the white rats 2 cc. each, intracardially. Two of the guinea pigs were injected by the intracardiac, the other by the intraperitoneal route, each receiving 1.5 cc. From patient No. 4, 6 cc. of blood was injected into each of 2 monkeys on May 12, 1939. Blood from patient No. 5 was injected in 10 cc. portions into each of 2 monkeys on May 3, 1940. The injections were made intravenously.

Two popliteal lymph nodes were removed from each of 6 of the rabbits. These were taken from the third to the eighth day, inclusive, after inoculation, so that continuous daily biopsies were available during that period. Sixteen of the 20 rabbits used in these experiments, and in that described below, were sacrificed for necropsy at intervals of 6, 7, 8, 9 and 11 days, respectively; 3 others were sacrificed 1 month after inoculation. The rats were sacrificed 4, 6, 8 and 11 days, and the guinea pigs 6 and 11 days, after injection. Sections were taken from the lymph nodes, parenchymatous organs, respiratory and gastro-intestinal tracts of all animals. All tissues, whether derived from biopsy or necropsy, were fixed promptly in 4 per cent aqueous solution of formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

An additional experiment, designed to repeat the work of Duval and D'Aunoy[3] who claimed to have increased the virulence of the virus of measles by animal passage, was performed as follows: On April 18, 1938, that is, 3 and 6 days after their inoculation, 2 of the rabbits which had been injected with the blood of patient No. 1, and 2 with blood from patient No. 2, were bled. The blood was "citrated," pooled and injected intravenously into 3 healthy rabbits, each of which received 6 cc. Seven days later blood was removed from each of these rabbits and injected into 3 fresh rabbits. A week later blood was taken from these and injected into 3 additional rabbits. Thus the agent received by the third group had been passed serially through three groups of rabbits. Two other rabbits were injected with blood from normal rabbits as a control. The animals used in this, as in all the other experiments, were kept under close clinical observation and a daily record made of their morning and eve-

ning temperatures for a period of 14 days after injection. Total and differential counts of the white blood cells were made.

From each of the 4 monkeys, an inguinal lymph node was removed immediately before injection and 5 additional nodes at intervals thereafter. Thus a lymph node was removed from monkey No. 1 on the fourth, sixth, eighth, tenth and eleventh days; from No. 2 on the third, fifth, seventh, ninth and eleventh days; from No. 3 on the third, fifth, seventh, tenth and twelfth days; and from No. 4 on the fourth, sixth, eighth, eleventh and fourteenth days after inoculation (Table I). The lymph nodes were fixed in 4 per cent aqueous solution of formaldehyde, embedded in paraffin and stained with hematoxylin and eosin. Several sections were cut from each node and examined microscopically. The monkeys were kept under close clinical observation for 1 month after inoculation, search being made daily for signs of enanthem, exanthem, conjunctivitis or coryza.

TABLE I

The Occurrence of the Giant Cell Reaction in the Lymph Nodes of Monkeys which Had Received Human Blood

Monkey No.	Date inoculated	Source of blood	Amount injected	Giant cell reaction in lymph nodes Days after inoculation											
				Control	3	4	5	6	7	8	9	10	11	12	14
1	5-12-39	Pt. No. 4	6 cc.	−		+		+		+		±	−		
2	5-12-39	Pt. No. 4	6 cc.	−	+		+		+		+		−		
3	5-3-40	Pt. No. 5	10 cc.	−	−		−		+			+		−	
4	5-3-40	Pt. No. 5	10 cc.	−		−		−		−			−		−

Results. None of the rats, guinea pigs or rabbits exhibited clinical signs indicative of measles and there were no significant changes in the temperature of the rabbits. The blood counts of the rabbits fluctuated within wide limits, but similar fluctuations occur in healthy rabbits.[38] None of the lymph nodes removed from the rabbits contained giant cells of the Warthin-Finkeldey type, nor were any seen in the tissues removed at autopsy from the rats, guinea pigs or rabbits. None of the monkeys developed coryza, conjunctivitis or exanthem during the 4-week period of observation. Monkey No. 3, which received 10 cc. of blood from patient No. 5, developed a small, whitish papule on the labial mucous membrane opposite the left lower central incisor on the sixth day after injection. This became hyperemic the next day

and disappeared 3 days later. It never showed the bluish central zone of a typical Koplik's spot, but was nevertheless somewhat suggestive of an enanthematous lesion. The animal was listless and had slight chills from the ninth to the eleventh day, inclusive, after inoculation. No lesion of an enanthematous nature was seen in any of the other monkeys.

Sections of the lymph nodes, removed from the monkeys before injection, showed no microscopic pathology other than slight localized hemosiderosis in monkey No. 2. Those removed after inoculation all revealed some degree of hyperplastic lymphadenitis. In addition, Warthin-Finkeldey giant cells were present near areas of reticulum hyperplasia and occasionally in the follicles of some of the lymph nodes taken from monkeys Nos. 1, 2 and 3. These giant cells varied considerably in appearance. Some, apparently recently formed, were small and appeared to be derived from monocytes which had multiplied by amitotic division (Figs. 1 and 2). Others were of such size as to be present in several consecutive sections cut approximately 6 μ thick. These had a variable number of pale, vesicular nuclei arranged in a cluster near the center of the cell, of which the cytoplasm was syncytial and faintly granular in character (Figs. 3 and 4). Some lymph nodes, removed from 2 to 6 days after the initial appearance of the Warthin-Finkeldey reaction, contained giant cells with hyaline cytoplasm and pyknotic nuclei (Figs. 5 and 6). These cells resembled megakaryocytes. These changes are interpreted as indicative of involution. None of the lymph nodes contained as many Warthin-Finkeldey giant cells as are commonly seen in human tissues. Occasionally, two were seen in close proximity (Figs. 3 and 4) and sometimes three (Fig. 7). The reaction lasts from 3 to 6 days, appearing as early as the third or as late as the seventh day after inoculation.

Discussion

On the basis of the clinical and histological data presented, white rats, guinea pigs and rabbits are not susceptible to the virus of human measles. Some monkeys, on the other hand, do appear to be susceptible.

The giant cell reaction described and illustrated in the lymph nodes removed from the monkeys, is almost identical with that

considered pathognomonic of impending measles in man. It was absent from the control nodes removed immediately before inoculation. It appeared after an incubation period and disappeared after an interval compatible with clinical experience with measles in man. Above all, the fact that no giant cells were seen in the lymph nodes removed from monkey No. 4, indicates that the reaction is not due to the injection of blood *per se*. It is interesting that none of the monkeys, with the possible exception of No. 3, exhibited clinical signs of measles. This may be accounted for in one of two ways: either the virus used by us was attenuated or the monkeys were relatively resistant. In either case it is not unlikely that the Warthin-Finkeldey giant cell reaction is a much more sensitive indication of infection than are clinical manifestations. In man, infection may be so mild that a diagnosis of measles cannot be made with certainty. This was true of one of the cases reported by Warthin,[22] and may be the explanation for the changes reported by Tomlinson.[33] It is planned to test two of the monkeys later for immunity. It is also planned to study tissues from chick embryos inoculated with blood from patients with measles. The present series of experiments is reported now in order to stimulate similar studies by others.

An additional point is worthy of emphasis. Occasionally the plane of a section passes tangentially through the wall of a small vessel. In such cases the nuclei of the endothelial cells appear clumped, producing a formation suggesting a multinucleate giant cell. It is rather easy to distinguish between the Warthin-Finkeldey and the fictitious giant cell. The nuclei of the latter are arranged in a much more orderly manner than those of the former and the lumen of the vessel becomes apparent in consecutive sections. The Warthin-Finkeldey giant cell, on the other hand, always shows its typical cluster of nuclei and never a lumen.

Summary

Blood taken from patients with measles was injected into white rats, guinea pigs, rabbits and monkeys.

The animals were kept under clinical observation and their tissues examined for Warthin-Finkeldey giant cells.

Histologically, there was no evidence that measles can be transmitted from man to white rats, rabbits or guinea pigs.

Blood originally drawn from patients with measles was passed serially through three rabbits and injected into normal rabbits. The latter showed no clinical or histological evidence of measles.

One monkey out of four injected with human blood developed a questionable enanthem 6 days after inoculation. This lesion disappeared spontaneously 3 days later. This animal appeared listless and had chills from the ninth to the eleventh day after inoculation. The other three monkeys exhibited no clinical signs of measles.

Giant cells were found in the lymph nodes of three of four monkeys injected with blood removed from two patients with mild attacks of measles. These giant cells resemble those described by Warthin and by Finkeldey in tonsils removed during the prodromal stage of measles.

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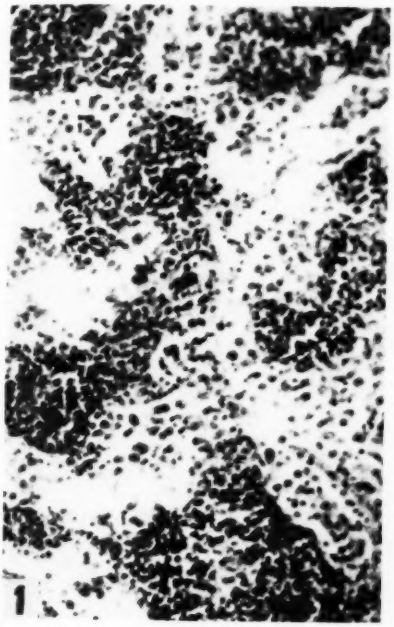
DESCRIPTION OF PLATES

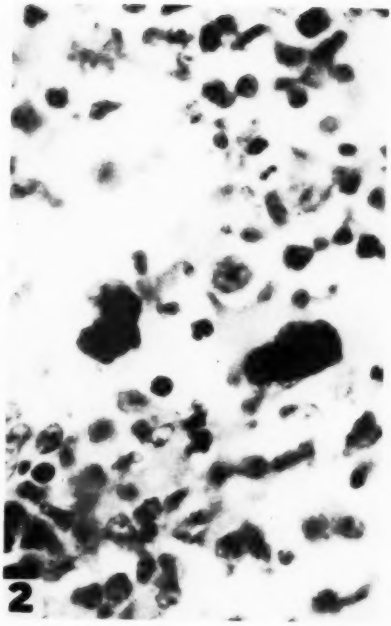
PLATE 36

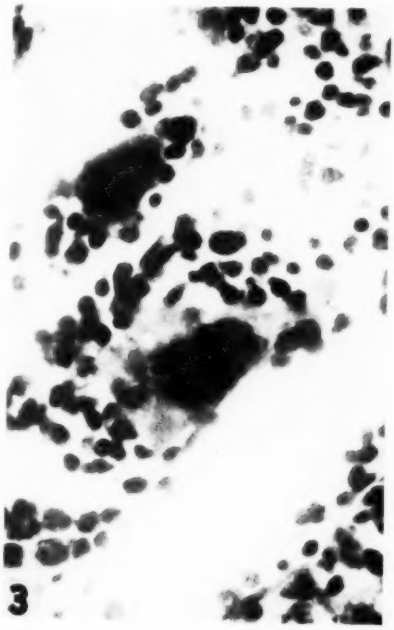
FIG. 1. Lymph node removed from monkey No. 2, 5 days after injection with 6 cc. of blood taken from patient No. 4. Near the center are two giant cells. There is a diffuse proliferation of monocytes and reticulum cells in the pulp. Hematoxylin and eosin stain. × 130.

FIG. 2. Higher magnification of a portion of the area included in Figure 1, to show the character of the giant cells and their probable origin from monocytes. × 440.

FIGS. 3 and 4. Lymph node removed from monkey No. 2, 9 days after injection. These photographs are made from consecutive sections, cut about 6 μ thick. There is a distinct difference in the configuration of the giant cells in the two photographs. The clumping of the nuclei and the syncytial character of the cells is very evident. Hematoxylin and eosin stain. × 460.







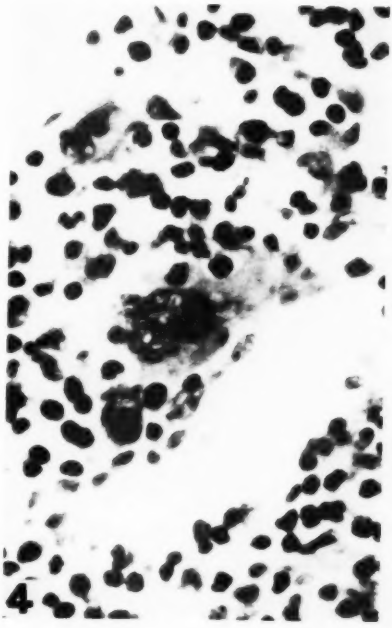


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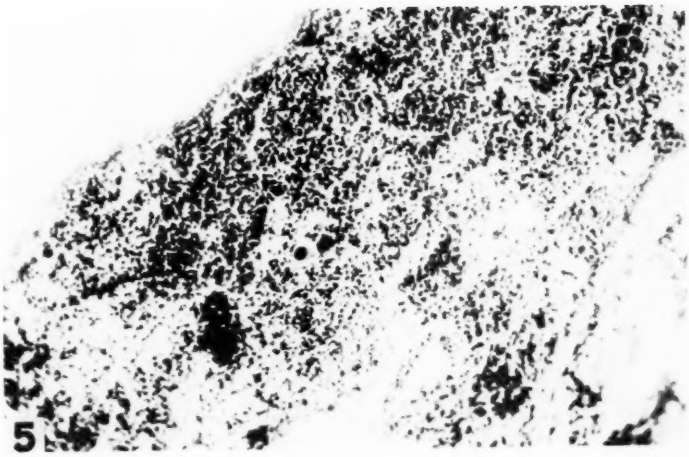
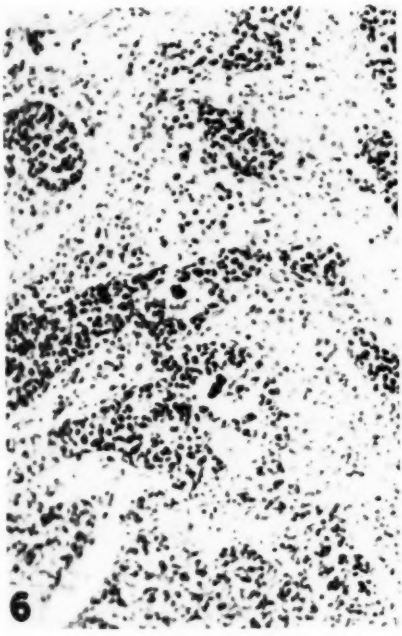
PLATE 37

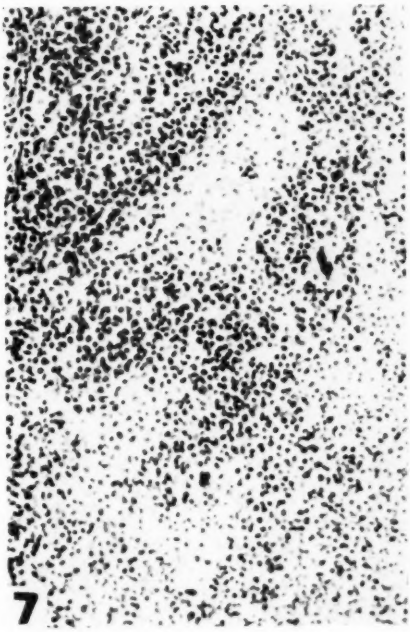
FIG. 5. From a lymph node removed from monkey No. 3, 10 days after injection with 10 cc. of blood taken from patient No. 5. The cytoplasm of one giant cell is hyaline and its nuclei are pyknotic. Hematoxylin and eosin stain. × 130.

FIG. 6. From a lymph node removed from monkey No. 1, 10 days after injection with 6 cc. of blood taken from patient No. 4. Below the center is an involuting giant cell with little cytoplasm and pyknotic nuclei. Hematoxylin and eosin stain. × 130.

FIG. 7. From a lymph node removed from monkey No. 3, 7 days after injection. Three giant cells are present in one field. The syncytial character of the giant cell near the center is apparent. Hematoxylin and eosin stain. × 130.







Gordon and Knighton Experimental Measles

A STUDY OF CHICK-EMBRYO-ADAPTED RABIES VIRUS*

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Prior to the first publication from this laboratory in 1939,[1] all attempts to establish rabies in chick embryos had been unsuccessful. Others, [2,3,4] as well as myself, had tried to induce rabies in the embryo but in all of these experiments inoculations had been made on the chorio-allantoic membrane. The advent of new technics for direct inoculation of the embryo [5] has made it possible to reinvestigate the chick embryo as an experimental host for rabies virus.

MATERIALS AND METHODS EMPLOYED IN ESTABLISHING RABIES IN CHICK EMBRYOS

The histories of three strains of rabies virus which were established in embryos are summarized in Table I.

The first strain used in this study was obtained from the brain of a dog dying of street rabies. An emulsion of the animal's brain was injected intracerebrally into Swiss mice, which developed weakness and paralysis on the seventh day and prostration on the eighth day. Some were killed on the eighth day and 0.05 cc. amounts of 10 per cent emulsion of the pooled brains were injected intracerebrally into day-old chicks. The chicks developed weakness and paralysis after 17 or 18 days; some were killed, and pooled brain emulsion was passed intracerebrally to other day-old chicks. Four passages in newly hatched chicks failed to cause any significant shortening of the incubation period or any change in the clinical course of the disease.

Pooled brains from the fourth generation in chicks were emulsified to 10 per cent strength and 0.05 cc. amounts were injected into the brains of embryos 13 days old. The technic for intracerebral inoculation of embryos is as follows: fertile eggs, after

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TABLE I
Histories of Three Strains of Chick-Embryo-Adapted Rabies Virus

	Strain C	Strain R	Strain H
Type of virus	street	fixed	human
Source of virus	dog brain	rabbit brain	human brain
Experimental hosts used in preliminary passages	mice day-old chicks day-old chicks day-old chicks day-old chicks	none	mice
Number of passages in embryos	65	25	3

13 days' incubation, are candled to locate the head and a square window 2 cm. in width is then cut in the shell as described by Goodpasture and Buddingh.[6] A ½ inch 27 gauge needle fitted to a 0.25 cc. syringe is used to inoculate the embryos. If the head is not accessible, the needle may be used to manipulate it but rough handling is to be avoided. Injection can be made anywhere in the brain, but in our experience the tectum is more easily inoculated. Once the head is exposed and the desired site located, a sudden short thrust will usually force the needle through the calvarium. After inoculation the window is ringed with a vaseline-paraffin mixture and closed with a coverglass.

Four days after inoculation one of the first generation embryos was killed and its brain passed to embryos 13 days old. Subsequent passages were made at intervals of 6 days. All inoculations were made in the manner described except that in later generations the volume of the inoculum was reduced to 0.03 cc. This strain, which is designated strain "C," has been carried through 65 generations in the embryo.

A second strain, established by direct inoculation of 10 per cent emulsion of rabbit-brain-fixed virus into the brains of embryos, has been maintained in this host for 25 generations. This strain is designated strain "R." The third strain was derived from a human case of rabies. An emulsion of human brain was inoculated intracerebrally in mice and pooled brains of prostrate mice served as the original inoculum for embryos. In establishing this strain it was necessary to use embryos 10 days old and to allow an incubation period of 10 days. This strain, which is designated

strain "H," has been passed only three times but histological study of brains of embryos of the third generation revealed changes characteristic of rabies.

Clinical Evidence of Rabies in Inoculated Embryos

Intracerebral inoculation resulted in death, presumably from trauma, in some of the embryos within 24 hours after inoculation but with proper technic this mortality should not exceed 30 per cent and it may be as low as 10 per cent. During the next 5 or 6 days, deaths were extremely uncommon. Some embryos died between the sixth and eighth days when they were 19 or 21 days old, and it is probable that these deaths were due to rabies; however, many, and in fact most, of the embryos survived considerably longer. Many embryos survived inoculation for 13 days and one lived for 14 days. Thus it is possible for an embryo to survive 5 or 6 days beyond the expected hatching date. Development was slowed to some extent but the embryos continued to exhibit rhythmic movements. In the embryos which went beyond the normal hatching date the yolk became inspissated and it was not incorporated within the peritoneal cavity. In my experience no embryo inoculated intracerebrally with rabies virus has ever hatched.

Pathological Changes Associated with Rabies in the Chick Embryo

No gross changes were observed until the third or fourth embryo generation when a mild hydrocephalus was noticed. This hydrocephalus, which was observed in each of the three strains, became much more severe in later generations. It was detectable as early as the third or fourth day and it increased rapidly after this time. The hydrocephalus was symmetrical and it was unassociated with any appreciable enlargement of the skull or thinning of the tables. The entire ventricular system was dilated and there was also an enlargement of the subarachnoidal spaces, especially those around the cerebellum. Symmetrical hemorrhagic necrosis of the tectum and of the forebrain appeared in later generations.

Microscopic examination revealed, in addition to the hydrocephalus, an hydromyelia. There were also encephalomalacia and

myelomalacia of a severe degree. Inflammatory cells in the central nervous system were few, consisting chiefly of large mononuclear phagocytes, but outside the central nervous system in the dorsal root ganglia, sympathetic ganglia and in the intrinsic ganglia of the various viscera, large collections of lymphocytes were seen.

The more specific microscopical lesions consisted of Negri bodies. These inclusions, stained by the Goodpasture method,[7] appeared as acidophilic, refractile, hyaline bodies, which varied greatly in size and in shape. In the larger bodies, vacuoles and basophilic inner bodies were demonstrable but the smaller ones were usually homogeneous. Characteristically, the inclusions were within the cytoplasm but many appeared to lie outside of the cells. These no doubt were Negri bodies situated far out in the processes of neurons or were released from the cytoplasm of disintegrated cells. These cytoplasmic inclusions were present throughout the central nervous system, in the retina and in various peripheral ganglia. They were present as early as 3 days and as late as 13 days after inoculation but they were most numerous on the sixth and seventh days. Neurons were affected chiefly but Negri bodies were also observed in ependymal epithelium. They were occasionally seen within the cytoplasm of large mononuclear phagocytes, presumably as a result of phagocytosis. No significant changes were found in the non-nervous tissue.

Negri bodies in the chick embryo are unusually numerous; most cells are involved and many cells contain several. This is especially true in the central nervous system and to a lesser degree for cells in the retina and in peripheral ganglia. The Negri bodies in the chick embryo are so numerous and so widely distributed that the diagnosis of rabies may be made from sections of any portion of the nervous system containing nerve cells.

Susceptibility of Chick Embryos to Rabies Virus Inoculated by Routes Other than the Intracerebral

All routine serial passages were made by intracerebral inoculations but other routes have been studied to some extent. In early generations attempts to produce an infection by inoculation of the chorio-allantoic membrane and by intramuscular inoculation

were unsuccessful. However, even in the earliest attempts (sixth generation) intra-ocular inoculation was always successful. In later generations infection was established by inoculations on the chorio-allantoic membrane, in the muscles of the thigh and in the yolk sac. Complete studies of the disease produced by these routes of inoculation have not yet been made but the end results appear to be the same regardless of the route of inoculation: hydrocephalus develops, Negri bodies are numerous and there is extensive destruction of nervous tissue. The incomplete studies of other routes indicate that the incubation period is somewhat longer when routes other than the intracerebral are used. The pathogenesis of the disease following these other routes of inoculation is being investigated.

Virulence of Chick-Embryo Virus for Various Host Species

Chick-embryo virus was inoculated into other species of animals in order to determine whether or not changes in its virulence for these hosts had occurred. These studies are summarized in Table II.

There are several points of interest which are not shown in this table. Strain C, which has been studied most carefully, was first reinoculated into mice after three passages in the embryo. These mice developed weakness and paralysis on the sixth or seventh day and died on the eighth day after inoculation. Sections from the brains revealed many Negri bodies and much perivascular exudate. From a clinical and pathological point of view these mice behaved as mice usually do when infected with street virus. Seventeenth generation virus was also put into mice. They became ill on the fifth or sixth day and died on the seventh or eighth day but they exhibited tremors and convulsions instead of weakness and paralysis. At autopsy very few Negri bodies were found and there was meager perivascular exudate. In this generation, therefore, there was definite evidence of alteration in the virus from a clinical as well as a pathological aspect.

Twenty-first generation virus was inoculated intracerebrally, subcutaneously and intramuscularly, using 3 mice in each group; all developed tremors and convulsions and 6 of the 9 died. However, 2 of the subcutaneously inoculated mice and 1 of those inoculated intramuscularly survived.

TABLE II
Inoculation of Other Host Species with Embryo-Adapted Virus

Strain C					Strain R				
Number of passages in embryo	Host		Route*	Results**	Number of passages in embryo	Host		Route*	Results**
	No.	Type				No.	Type		
3	6	mice	icer.	8, 8, 8, 8, 8, 8.	4	6	rabbits	icer.	5, 7, 7, 7, 7, 8.
17	5	mice	icer.	7, 7, 7, 7, 8.	14	3	dogs	icer.	5, 5, 5.
21	3	mice	icer.	7, 8, 9.					
	3	mice	sbc.	10, S, S.					
	3	mice	imus.	8, 10, S.					
22	6	rabbits	icer.	10, 13, 16, S, S, S.					
26	6	dogs	icer.	9, 9, 9, 10, 10, 10.					
29	13	rabbits	icer.	11, 11, 12, S, S, S, S, S, S, S, S, S, S.					
43	6	mice	icer.	7, 9, 10, 11, 12, 13.					
	3	dogs	icer.	9, 9, 14.					

* icer. = intracerebral; sbc. = subcutaneous; imus. = intramuscular.
** Figure indicates day of death. S indicates that the animal became sick but survived.

Encouraged by these survivors, 22nd generation virus was inoculated intracerebrally into 6 rabbits and they developed fever, extreme ataxia and incoördination. Because of this they were unable to eat but if food was held for them they were able to chew and swallow it. However, no attempt was made to maintain their nutrition. One animal appeared prostrate on the tenth day but was not paralyzed. This animal was killed and sections of its brain showed a few Negri bodies and some exudate. One rabbit died on the 13th day and another on the 16th day. Histological examination of their brains revealed no Negri bodies and a 10 per cent emulsion of brain from the rabbit which died on the 16th day failed to produce rabies when inoculated intracerebrally in mice. The remaining animals gradually recovered except for some residual ataxia.

Twenty-sixth generation virus inoculated intracerebrally in dogs produced a uniformly fatal disease characterized by fever, tremors, ataxia and convulsions. Twenty-ninth generation virus inoculated intracerebrally in 13 rabbits produced symptoms of mild fever and ataxia in all of them, but only 3 died. Forty-third generation virus given to mice and dogs produced a fatal disease similar to that already described for these species.

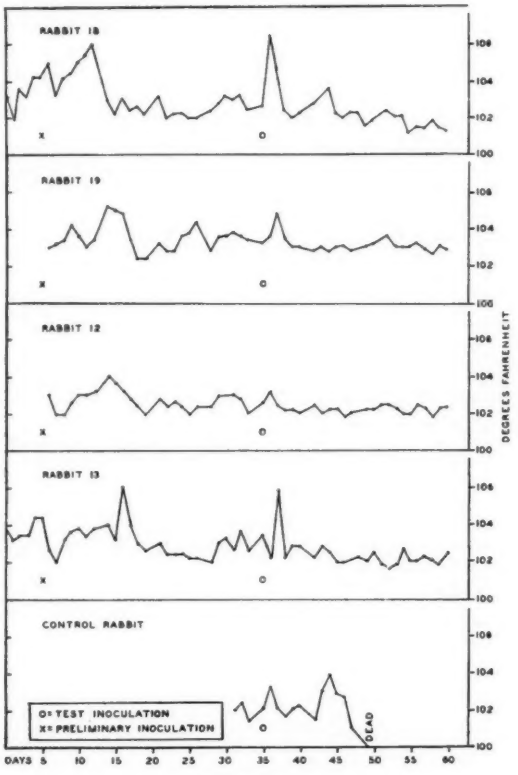
Very little work has been done with strain R but it was highly virulent for dogs and rabbits, producing a rapidly fatal disease characterized by fever and flaccid paralysis.

Immunity Tests on Survivors

The 3 mice which survived inoculation of the 17th generation of strain C were tested for immunity by an intracerebral inoculation of 0.03 cc. of 10 per cent mouse-passage virus. This procedure was not followed by any detectable change in the test mice but control mice died in 8 days.

Four of the rabbits surviving the intracerebral inoculation of the 29th generation of strain C were also tested for immunity by the intracerebral inoculation of 0.5 cc. of 5 per cent emulsion of two strains of rabies virus: one was rabbit-fixed virus and the other was mouse-passage human virus. Temperature charts of the rabbits which received the original and test inoculation and of 1 control rabbit which received 0.5 cc. of 0.05 per cent mouse-

passage virus are shown in Text-Figure I. All of the test rabbits survived the inoculation, whereas the control animals receiving 0.5 cc. of 0.05 per cent emulsion died of rabies. The rise in temperature which occurred after the reinoculation of 3 of the test



TEXT-FIG. 1.
Temperature charts of rabbits receiving the original and test inoculations, and of one control rabbit.

animals requires explanation. The animals, both test and control, were inoculated and within 15 minutes the temperatures were taken. A similar rise in temperature, immediately following intracerebral inoculation, has been noted in normal animals, so it seems likely that this fever is simply a reaction to trauma and not an immunity response.

Discussion

These experiments are important from at least two points of view.

1. They provide a new experimental host in which rabies may be studied. The chick embryo is a unique host in many respects but its chief value in the experimental study of rabies lies in its ability to withstand a massive inoculation of virus for as long as 14 days. Since evidences of infection of the central nervous system are present as soon as 3 days after inoculation, this means that the embryo has had rabies for about 11 days, during which time the greater portion of the nervous system has been destroyed. This remarkable ability of the embryo to withstand the infection much longer than other hosts makes it possible to study the more mature lesions of rabies. These mature lesions are remarkable because of their widespread distribution and their extremely destructive nature. Six days after intracerebral inoculation the lesions occur throughout the central nervous system and in the retina and it is difficult to find any peripheral ganglion which does not show evidence of infection. In addition there is extensive destruction of nerve tissue which is associated with the development of a marked hydrocephalus and hydromyelia as well as encephalomalacia and myelomalacia. It seems likely that the hydrocephalus is due, in part at least, to the destruction of undifferentiated elements which in normal development would have continued to multiply and differentiate to form the mature elements of the nervous system. The destruction of these immature, actively proliferating cells would undoubtedly have a tremendous effect upon subsequent development of the brain and cord. It is of course obvious that destruction of fully matured nerve cells is also important in the genesis of this lesion. It is unlikely that the hydrocephalus develops on the basis of obstruction: it did not occur in the first three or four generations; it has not been observed (except in extremely rare instances) when noninfectious brain was inoculated intracerebrally; it is not associated with enlargement of the skull; and it develops when virus is inoculated by routes other than intracerebral.

2. The experiments indicate that intracerebral passage of rabies virus in the chick embryo is associated with a profound alteration

of the pathogenicity of this virus for the rabbit and the mouse. This alteration is of such magnitude that it is possible to produce a self-limited, nonfatal disease in rabbits by intracerebral injection and in mice by subcutaneous or intramuscular injection of embryo-passage virus. Furthermore, tests show conclusively that recovery from the mild disease is followed by the development of a solid immunity to intracerebral inoculation of rabbit-fixed virus and mouse-passage virus.

The immunizing properties of chick embryo virus are important from both a theoretical and a practical point of view. The development of an actively immunizing strain of rabies virus, whereby immunity resulting from actual infection may be produced, would make it possible to study the immune response of animals to the complete rabies antigen. Heretofore this has been impossible since the immune response to vaccines has been dependent upon the pre-formed antigen introduced during vaccination, and furthermore this antigen has been altered by the inactivation of most vaccines. From a practical point of view such a strain of virus would be an ideal vaccine since it would be possible to immunize the animal with a single dose of virus which actually produced a mild infection. It would, of course, be essential that the mild disease produced in this fashion be nontransmissible in nature.

At present the embryo-passage virus is still highly virulent for dogs but it is hoped that repeated passages in the embryo will bring about alterations in virulence for dogs comparable to those which have already occurred for rabbits.

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DESCRIPTION OF PLATES

PLATE 38

FIG. 1. Gross photographs of head of an embryo inoculated intracerebrally on the 13th day of incubation with 22nd generation of strain R. Killed 6 days after inoculation. The lower photograph of the dorsum of the skull shows an hydrocephalus of mild degree and in addition two black areas in the forebrain. These are areas of hemorrhagic necrosis. The upper photograph is a longitudinal section a few millimeters to the left of the midline. It shows the forebrain to be somewhat smaller than normal. The major portion of the forebrain is quite dark due to hemorrhagic necrosis. Immediately behind the forebrain is seen the moderately dilated lateral ventricle. Below this ventricle lies the tectum. It is not necrotic but its ventricle is greatly dilated.

FIG. 2. Gross photographs of the head of an embryo inoculated intracerebrally on the 13th day of incubation with 42nd generation of strain C. Killed 6 days after inoculation. The lower photograph shows the dorsal aspect of the skull. Note the dark portion which lies behind the small lobes of the forebrain. These are dilated ventricles. The upper photograph shows a longitudinal section about 3 mm. to the left of the midline. The small forebrain lies just above and a little behind the eye. Immediately behind is the greatly dilated ventricle. Below the ventricle lies the tectum, part of which is grayish in color and part black. The black portion is the site of hemorrhagic necrosis.

FIG. 3. Photomicrograph of section of embryo head in a plane 1 or 2 mm. to the right of the midline. Embryo inoculated intracerebrally with 45th generation of strain C. Killed 6 days after inoculation. The small forebrain lies above the eye. A thin strip of nervous tissue and meninges extends backward from the superior portion of the forebrain. This strip parallels the skull for a distance of about 15 mm. and then turns downward and finally forward again to merge with the base of the brain. Behind the small forebrain lies the greatly dilated lateral ventricle which is bounded above and behind by the thin strip of tissue already described. The portion of brain which extends downward and backward from the forebrain appears extremely pale. Only a thin shell of forebrain remains posteriorly, and immediately beneath this is seen the tentorium cerebelli. The cerebellum with its thin, simple convolutions surrounded by the greatly dilated subarachnoidal space is well illustrated. The fourth ventricle is greatly dilated. The choroid plexuses of the fourth ventricle and of the lateral ventricle are clearly shown. × 4.

FIG. 4. Photomicrograph of head of normal embryo of the same age as the one in Figure 3. This section is taken in almost exactly the same plane as that in Figure 3 and is included for comparison. × 4.

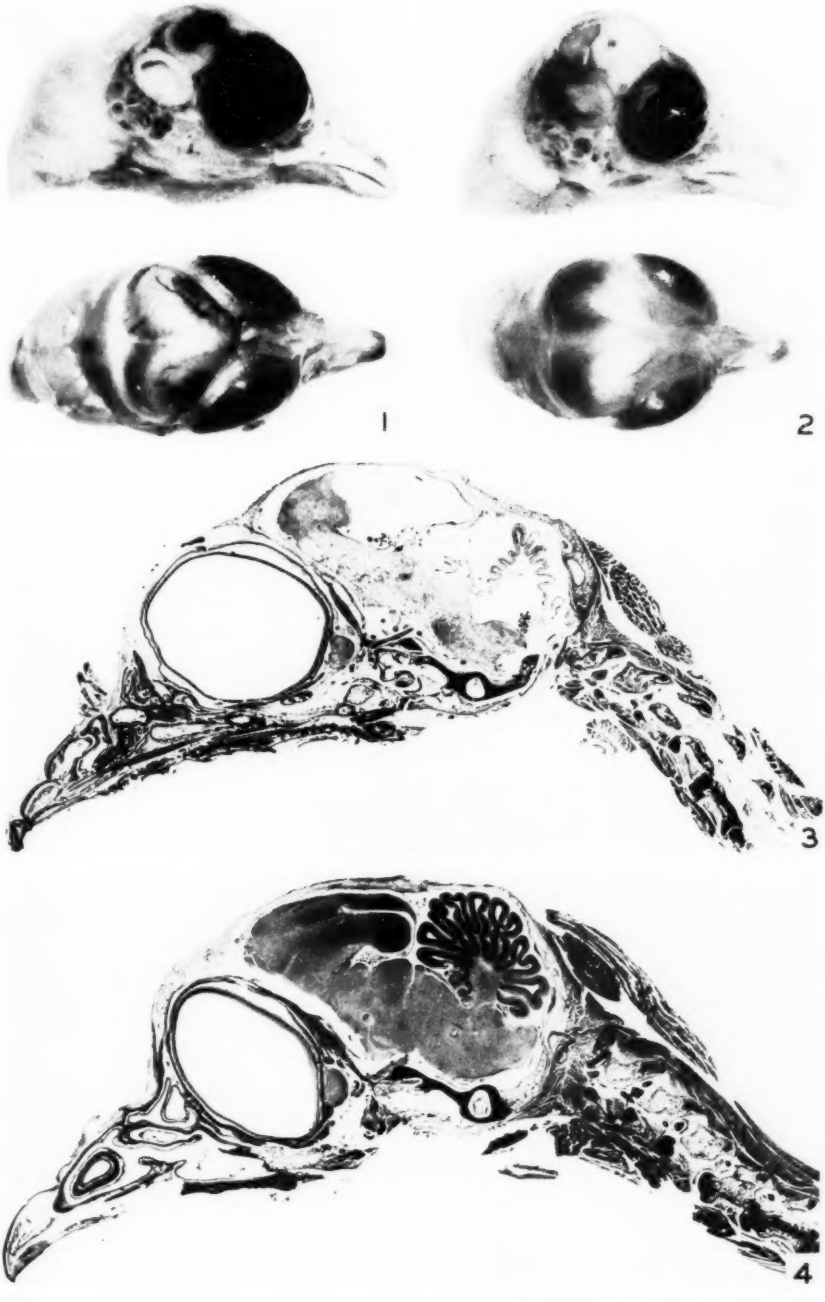
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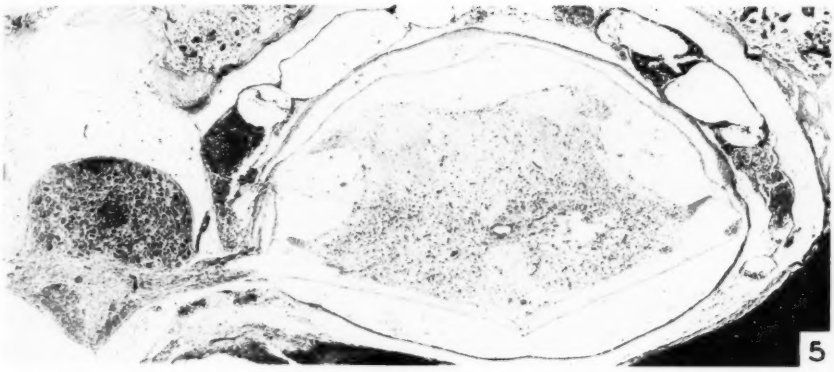
PLATE 39

FIG. 5. Low power photomicrograph of cord and dorsal root ganglion from embryo inoculated intracerebrally with 17th generation of strain C. Killed at 6 days. Note the wide subarachnoidal space and the rarefied appearance of the cord itself. In the dorsal root ganglion are seen two large accumulations of round cells. × 40.

FIG. 6. Photomicrograph of the same section used for Figure 3. The field photographed lies directly behind the eye. This photomicrograph shows the extreme degree of encephalomalacia. × 40.

FIG. 7. Higher power photomicrograph of a field similar to that shown in Figure 6. Note the extreme rarefaction of the tissue. Blood vessels are numerous and a few cells persist around them, but the intervening tissue is almost completely destroyed. Inflammatory cells are notably few. × 65.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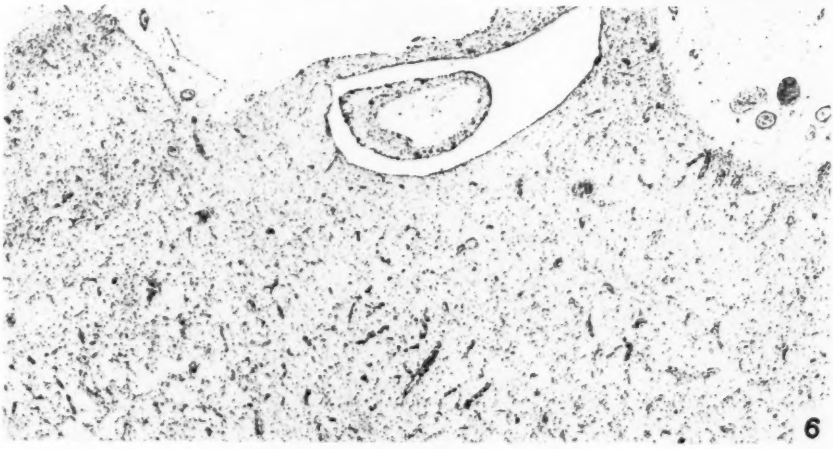
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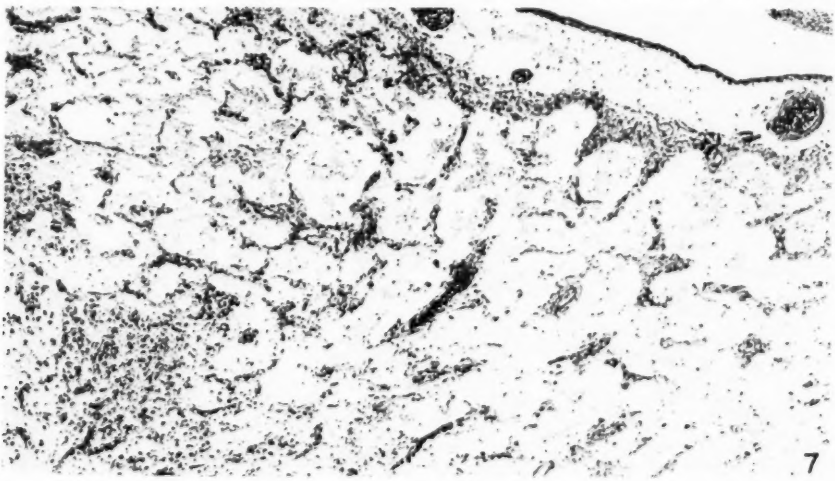
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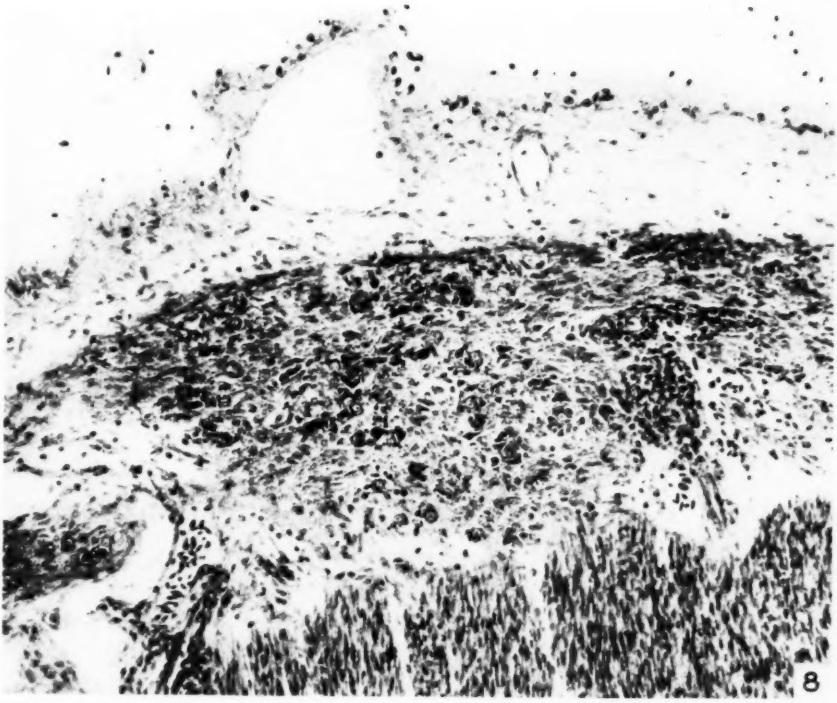
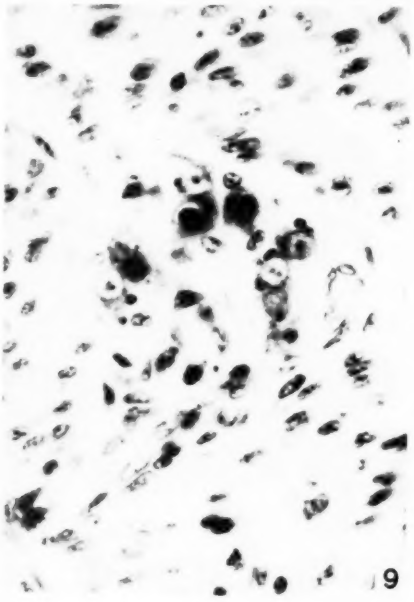
PLATE 40

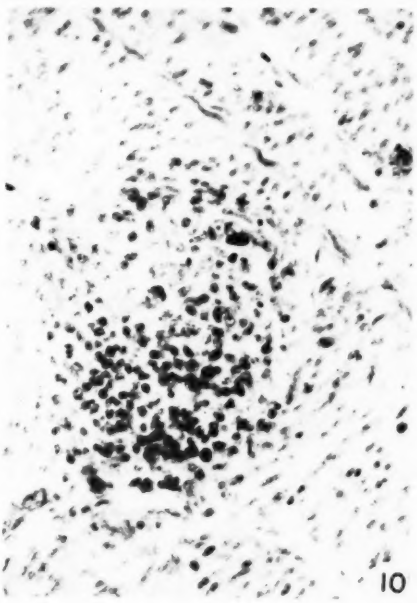
FIG. 8. A photomicrograph of a ganglion just beneath the serosa of the gizzard of an embryo 6 days after an intramuscular injection of 60th generation of strain C. The large black masses are Negri bodies. There is also some necrosis of nerve cells and some lymphocytic infiltration of the ganglion. × 120.

FIG. 9. Photomicrograph of small ganglion within the muscular layer of the gizzard of a chick embryo 6 days after an intracerebral inoculation of 17th generation of strain C. There are numerous Negri bodies within the cytoplasm of nerve cells. Halos surround several of the inclusions. There is no lymphocytic infiltration and very little necrosis. × 800.

FIG. 10. Another ganglion from the gizzard of the same embryo showing a few Negri bodies in the cytoplasm of neurons and a rather massive infiltration of round cells. × 350.







Dawson

Chick-Embryo-Adapted Rabies Virus

Plate 41

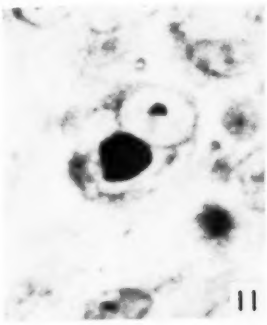
Fig. 11. Large Negri body in cytoplasm of cerebral neuron of chick embryo. × 1500.

Fig. 12. Neuron in chick embryo brain showing vacuolated Negri body in cytoplasm. Carbol-aniline-fuchsin methylene blue. × 1500.

Fig. 13. Negri body in cytoplasm in neuron of sympathetic ganglion of chick embryo. × 1500.

Fig. 14. Several nerve cells which contain Negri bodies. × 1500.

Fig. 15. Retina of chick embryo showing Negri bodies in all layers except the rods and cones. (Negri bodies occur in the rods and cones but they are not very numerous.) × 1500.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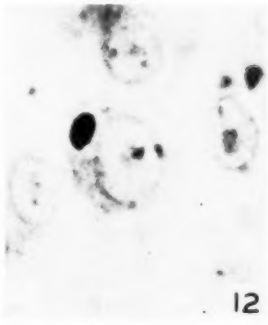
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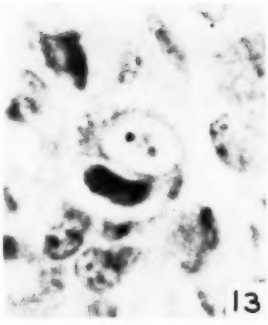
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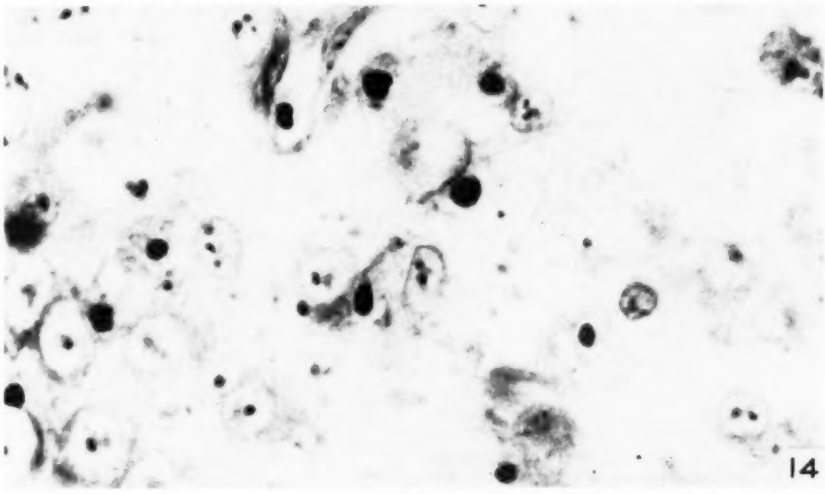
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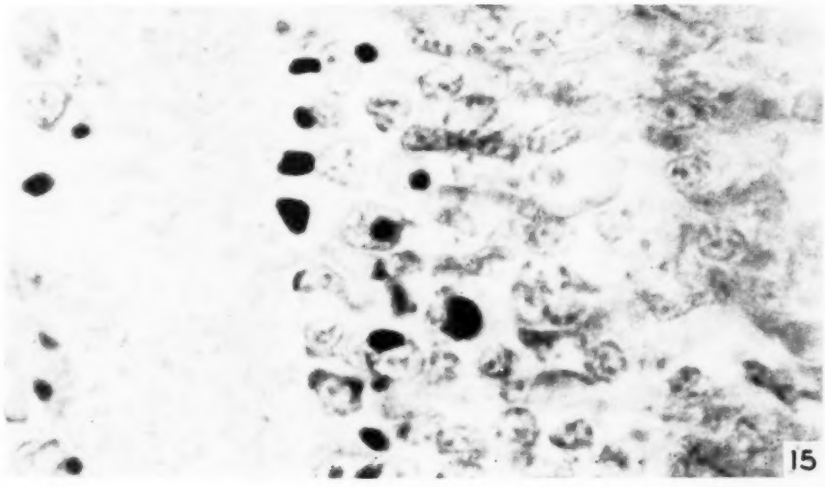
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EFFECTS OF SYNGENESIOTRANSPLANTS AND OF EXTRACTS OF THE ANTERIOR LOBE OF THE BOVINE HYPOPHYSIS ON THE AGE CHANGES IN THE LONG BONES AND JOINTS OF MICE *

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In previous investigations we [1,2] have shown that implants and extracts of the bovine anterior pituitary gland (1) increase proliferation, (2) induce degenerative changes in the epiphyseal and articular cartilage, and (3) stimulate the deposition of bone in the skeleton of the guinea pig. Under apparently identical experimental conditions, either the processes of proliferation or those of degeneration and ossification of the cartilage predominated. Gigantism was not observed in connection with either mode of reaction. Yet, the predominance of proliferation caused a temporary retardation of skeletal ageing, whereas an acceleration was noted if degenerative changes and ossification were more pronounced than growth of cartilage. If the anterior hypophyseal hormone was administered to young animals, the tendency of the cartilage to proliferate was stronger than in older guinea pigs under corresponding conditions; conversely, the tendency of the cartilage to degenerate was the greater the older the animals were when the treatment was begun.[3] But, the age factor could be only one of several which might be responsible for these different modes of reaction in the cartilage.

In a further attempt to clarify this problem, we analyzed the changes in bone and cartilage taking place under the influence of injections of extracts of the bovine anterior hypophysis as well as of homeo- and syngenesiotransplants of pituitary glands in closely inbred strains of mice.

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Material and Methods

The mice which served in these investigations and which had received transplants of the anterior hypophysis were the ones which had been studied previously by Loeb and Kirtz [4] as to the effect of these transplants on the growth and carcinomatous transformation of the mammary glands.

Transplants of the Anterior Lobe of the Hypophysis

Most of these experiments were carried out in virgin A mice, but also some mice of strains C_{57}, C_3H, and CBA were used. Seventy-six mice, all females except two, received three or four, and in three instances five, transplants of the anterior lobe of the hypophysis of litter mates, irrespective of the sex of the latter. If the litters were too small, additional pituitary glands of unrelated mice of the same strain were transplanted. As a rule the mice were 1½ to 2 months old at the time of transplantation, but in four instances the mice had reached the age of 3 and 3½ months. The transplants were allowed to remain in the host for 1 and 2 weeks, and for 1, 2, 3, 5, 6, 8, 9, 10, 11, 12 and 14 months, at the end of which periods the animals were sacrificed (Table I).

Table I

The Duration of the Experiment, the Number of Transplanted Glands and the Number of Mice in the Various Strains

Duration of experiment	Number of mice	Strains									
		A			C_{57}			C_3H		CBA	
		No. of glands used			No. of glands used			Glands used		Glands used	
		3	4	5	3	4	5	3	4	3	4
1 week	5	2	1		1	1					
2 weeks	5	1	2		2						
1 month	6	3	1		2						
2 months	6	2	2		1	1					
3 months	6	3	1		1	1					
5 months	7	2	3		1	1					
6 months	3	1	1	1							
8 months	3	1	1					1			
9 months	14	4	2		2	2		1	1	1	1
10 months	4	2	2								
11 months	5	1	3	1							
12 months	4							2	2		
14 months	8	3			2	2	1				
Total	76	25	19	2	12	8	1	4	3	1	1

Injections of Extract of Bovine Anterior Hypophysis

Thirty-three mice, thirty females and three males, belonging to strains C57, A, C_3H and D, received intraperitoneal injections of 0.1 or 0.2 cc. of freshly prepared bovine anterior hypophyseal extract on 4 or 5 days of the week. The age of the animals varied between 4 weeks and 2 months at the beginning of the injections, which were continued for periods of 1 and 2 weeks, and for 1, 3, 4, 6, 9, 10, 11, 13, 14, 15 and 16 months. The animals were killed 1 or 2 days following the last injection (Table II).

TABLE II

The Length of Time during which Injections of Anterior Pituitary Extract Were Made, and the Number of Mice of the Various Strains Used

Duration of experiment	Number of mice	Strains			
		C57	A	C_3H	D
1 week	4	2	2		
2 weeks	2		2		
1 month	2		2		
3 months	3	1	2		
4 months	2			1	1
6 months	2			2	
9 months	2			2	
10 months	1				1
11 months	4			2	2
13 months	2	2			
14 months	2	2			
15 months	5	4			1
16 months	2				2
Total	33	11	8	7	7

Specimens of the knee joint and dorso-ventral sections were prepared for microscopic study as described previously.[5]

MICROSCOPIC EXAMINATION

1. Normal Mice

The age changes taking place in the long bones during the life span of normal mice have been previously reported by us.[5] We may restate briefly, that as far as these ageing processes are concerned, three periods could be distinguished: during the first, the predominant processes were proliferation of cartilage and apposition of osteoblasts; during the second, degeneration, calcification and ossification of the cartilage cells and matrix; and during the third, absorptive processes in the skeletal tissues. Remarkable

differences were found to exist in different strains of mice as to the intensity and rapidity with which these changes took place. In D mice they progressed at a faster rate than in C_3H mice, and in C_3H mice, more rapidly than in A mice. In C57 and CBA mice skeletal ageing proceeded more slowly than in either A, C_3H or D mice. Breeding also accelerated the course of the age changes in mice belonging to strain C57. In C_3H mice, and to a certain extent also in D mice, more bony tissue was present during the first two periods of skeletal development than in C57 or CBA mice. Furthermore, in old mice, hypertrophy and retrogressive changes in the articular cartilage could be observed, the incidence of these processes again being higher in A, D and C_3H mice than in C57 or CBA mice.

II. Effects of Syngenesiotransplants of the Anterior Lobe of the Hypophysis

A. Zone of Endochondral Ossification. In mice 7 to 12 weeks old, which had received transplants 1 or 2 weeks previous to autopsy, the growth zones were definitely narrower than in normal animals of the same strain and age (Figs. 1 and 3). This narrowing was due to a decrease in the number as well as in the size of the individual cartilage cells. In the epiphyseal disk of the upper tibia only 1 or 2 hypertrophic cartilage cells were present in one column instead of 2 or 3, as is normal for this age; the number of columnar cartilage cells was 5 or 6, which is slightly below the normal figure of 6 or 7. The cartilaginous ground substance was increased in amount and strongly calcified; its fibrils were thickened. The resting and the columnar cartilage cells showed less proliferation than normally, mitoses being scarce or absent. The largest hypertrophic cartilage cells were smaller than the corresponding cells under normal conditions. Thus, 1 or 2 weeks after transplantation and at an age of 8 to 9 weeks, the appearance of the epiphyseal disk of an A mouse was similar to that of an untreated A mouse 3 to 4 months old.

The subepiphyseal zone, in which the replacement of the cartilage by bone and the formation of the osseous trabeculae take place, became increasingly narrowed, and instead of well vascularized bone marrow, a poorly vascularized fibrous tissue was

found in this layer. The calcareous spurs left over from the breakdown of the calcified cartilage capsules were covered by numerous actively proliferating epithelioid cells. The majority of these cells acted as osteoblasts, whereas only a few of them produced, by coalescence, osteoclastic giant cells. Thus, since more bony substance was deposited than was absorbed, the trabeculae were more numerous, thicker and more mature than is normal for this age and strain and they came to adjoin closely, with a broad base, the hypertrophic zone of the epiphyseal cartilage, whereas normally the formation of trabeculae takes place more distally in the metaphysis.

These conditions remained more or less stationary for the next 2 to 2½ months. In A mice, 5 months old, which had received anterior pituitary transplants at the age of 2 to 3 months, the epiphyseal growth zones had become still narrower (Fig. 5), the cell count having fallen to 1 or 2 hypertrophic and 4 or 5 columnar cells, whereas the corresponding normal figures are 2 or 3 hypertrophic and 6 or 7 columnar cartilage cells. Slight mitotic proliferation of the epiphyseal cartilage could also be observed, whereas proliferation of the epiphyseal cartilage ceases normally at the age of 4 months. Furthermore, marked retrogressive changes were not seen in the cartilage of the growth zones in bearers of pituitary transplants at this age, although they were present at this time in normal mice. Likewise, in transplant-bearing mice 6 months old the retrogressive changes were less pronounced than in normal mice at the age of 4 months. Consequently, the appearance of the epiphyseal disk of a mouse 5 months old which had received transplants was similar to that of a normal mouse 3 to 4 months of age. In the subepiphyseal zone, the production of bone around the trabeculae predominated over processes of resorption, and the formation of the transverse bony plate, which normally begins at the age of 4 months, was not seen in transplant-bearing mice 5 months old.

From 5 to 9 months subsequent to transplantation, the epiphyseal cartilage underwent an intensified and more rapid degeneration and calcification. Numerous amorphous plugs had taken the place of several disintegrated cartilage cell rows. These plugs subsequently underwent calcification or ossification, or both. Between these areas scanty remnants of sclerosed and calcified

euhyaline cartilage remained. In the subepiphyseal zone, the resorption of the osseous trabeculae became more pronounced and the formation of the transverse osseous plate underneath the epiphyseal cartilage had set in and made progress. The bony lamella was thicker than in normal mice of any age and strain (Fig. 6). The condition of the epiphyseal plate in these mice was comparable to that of normal animals in the second year of life, but more bone had been laid down than in such mice and there was greater destruction of the cartilage. In mice of 11 months and older, in which the transplants had been allowed to remain for 9 or more months, the solution of the bony plate was accelerated, due to the increased activity of capillaries and other elements of the bone marrow advancing from the metaphysis in the direction towards the epiphysis. Thus, perforations of the ossified epiphyseal plate were initiated, which were at first narrow and became, subsequently, more or less widened. Thus a degree of epiphyseodiaphyseal union was produced, which was farther advanced than that of corresponding normal mice.

B. Joint. One or 2 weeks following transplantation the osseous border lamella, which separates the cartilaginous covering of the joint from the epiphyseal bone marrow, was thickened; the bone cells were small and their nuclei and cytoplasm were dense; the line of calcification which ordinarily traverses the zone of hypertrophic cartilage had advanced farther proximally into the transitional zone. One month after transplantation a softening of the cartilaginous matrix and an increased proliferation and enlargement of the cells of the transitional zone became noticeable. Two or 3 months following transplantation the ground substance was markedly swollen, the cells of the sliding and transitional zones had undergone hypertrophy and a pronounced amitotic and, sometimes, mitotic division. Simultaneously, pyknosis, karyorrhexis and karyolysis occurred in other cells of this area. Five months after transplantation and at still later periods, the proliferative and retrogressive changes had made farther progress (Fig. 9). An outgrowth of cartilage cells and a desquamation of liquefying hypertrophic cells into the cavity of the joint had occurred, leading to irregularities of the articular surface and, in places, to an exposure of the bone underneath the cartilage (Fig. 10). This bony tissue was subsequently absorbed and rarefied progressively

by capillaries growing out from the epiphyseal bone marrow and penetrating deeply into the bony lamella. Edematous swelling, as well as proliferative and degenerative changes in the cells, had involved also the capsule and the ligaments of the joint (Fig. 11). From these combinations of growth and retrogressive processes severe arthropathic lesions resulted. In mice in the second year of life, organization of degenerated areas took place through ingrowth of vascularized connective tissue.

C. Diaphysis. During the first months following transplantation the periosteal connective tissue was dense and it contained thickened collagenous fibers. The surface of the cortex was smoother than in normal mice of the same age. The number of osteoblastic epithelioid cells on the periosteal as well as on the endosteal surface of the compacta was greater than that of osteoclastic giant cells. Therefore, the apposition of bone was more accentuated than was the absorption. The vascular canals traversing the bony shaft were narrow, the osteocytes were small, there was a large amount of osseous substance, and the cementing substance between the osseous lamellae was intensely calcified. Thus the compacta was firmer, thicker and more mature than is normal at this age. From 2 to 5 months following transplantation, the vascular canals became enlarged as compared with the condition found at the earlier stages, while the osteoblasts were somewhat less numerous. There was now an increase in the intensity of the absorptive processes and the compact bone appeared, therefore, only slightly thickened as compared with normal conditions at this age. After 6 to 12 months, the vascular and lacunar resorption had made still farther progress, while the new production of bone had not progressed to the same extent. In the second year of life the processes of solution predominated definitely over those of apposition and consequently a thinning of the compact bone ensued which surpassed in degree that of normal control animals.

The bone marrow was rich in cells during the first year of life. Toward the end of the first year, however, there was an increase in the amount of fat tissue and fibrous connective tissue, noticeable particularly in the epiphyseal marrow. In untreated mice similar changes were found only in the second half of the second year of life.

III. Effects of Bovine Anterior Hypophyseal Extract

A. Zone of Endochrondral Ossification. In mice 7 to 9 weeks old, after receiving injections of anterior hypophyseal extract for 1 to 2 weeks, the chondromucoid ground substance of the epiphyseal cartilage was loosened and swollen. The growth zones showed a regular columnar arrangement (Fig. 2). In the resting cells, and particularly in the columnar cartilage cells, mitoses were found more frequently than is normal for this age. The ordinarily flat columnar cartilage cells were enlarged and more vesicular, indicating their accelerated transformation into cartilage cells of the hypertrophic type. On the other hand, the layer of fully mature and intensely calcified hypertrophic cartilage cells was narrowed, as compared with the normal condition. This was due to the intensified breakdown of the hypertrophic cartilage cells caused by the rapid advance of capillaries from the bone marrow. The average number of hypertrophic cartilage cells in one cell column in the epiphyseal disk of the upper tibia had decreased from the normal number of 2 or 3, to 1 or 2, whereas the columnar cartilage cells, although they were enlarged and vesicular, were present in their normal number of 6 or 7.

After a series of injections extending over a period of 1 month, the cartilaginous matrix had become denser and more abundant and larger amounts of calcium had been laid down, particularly in and around the hypertrophic cartilage cells. The growth zones had become narrower than is normal for this age. In one cartilage cell column only 1 or 2 hypertrophic cells were counted, whereas the number of columnar cartilage cells had decreased to 5; still, the latter cells continued to proliferate, frequently by mitosis.

In these early stages the subepiphyseal zone contained many congested capillaries. Round epithelioid cells of the bone marrow underwent mitotic proliferation and arranged themselves in a beadlike manner along the calcareous spurs left over from the breakdown of calcified cartilage cell capsules. Subsequently, these osteoblasts became smaller; they were surrounded by osseous substance and were converted into osteocytes. Other epithelioid cells coalesced and formed osteoclasts, but these cells were not very numerous. After injections had been given for 1 month,

some of the osteoblasts began to arrange themselves in a transverse line close to the layer of hypertrophic cartilage cells. This represented an initial stage in the formation of the transverse osseous plate which in untreated animals was not seen before the age of 4 months.

In mice 5 months old, after injections for 3 months (Fig. 4), the number of cartilage cells in one column and the width of the growth zones were practically unchanged as compared with conditions seen after 1 month of injections. However, the cartilaginous ground substance had become still more abundant, sclerosed and pre-osseous, causing a shrinkage of the columnar and hypertrophic cartilage cells. In some areas degenerative changes had affected whole cartilage cell rows, and amorphous masses were found in their place. The latter became calcified and ossified, and numerous fairly thick, partly osseous, partly calcified, plugs traversed the epiphyseal disk in the direction from the metaphysis toward the epiphysis. The condition was as far advanced as in normal mice of the same strain 10 to 12 months old.

In the subepiphyseal layer, solution processes now predominated, causing a gradual corrosion and thinning of the osseous plate which separated the epiphyseal cartilage from the diaphyseal bone marrow. The longitudinal trabeculae were similarly affected, and finally disappeared.

After 4 months of injections, and to a more marked degree after 6 months and later, the growth zones were more and more narrowed, the cartilaginous matrix was increasingly sclerosed and osseous, and the columnar arrangement of the densely calcified cartilage cells became indistinct. In all strains studied, calcification and bone formation were accelerated as compared with conditions in normal mice of corresponding age. Thus, in a D mouse 6 months old, which had received injections for a period of 4 months, the main body of the epiphyseal cartilage was replaced by bone; wide gaps in the epiphyseal disk indicated an advanced stage of epiphyseo-diaphyseal union which had progressed even farther than it had in noninjected D mice in the second year of life. Similarly, in C57 mice 15 to 19 months of age which had been injected for 13 to 15 months, all or at least the greater number of the cartilage cells had been ossified, and in the most advanced cases the epiphyseal plate had disappeared. Thus a

complete epiphyseo-diaphyseal union in the femur (Fig. 7) as well as in the tibia was accomplished, a condition which had not been observed in normal mice of this strain even in the third year of life. In other instances, however, the epiphyseo-diaphyseal union had not made much progress beyond the stage present at about 1 year of age. But even here it was always farther advanced than in corresponding control animals.

B. Joint. As early as after 1 week of injections, but still more so after 2 weeks or 1 month, a marked proliferation of the cells of the sliding and transitional zones was seen in the majority of cases. The cells multiplied frequently by mitosis, but more commonly by amitotic division. They also increased in size, both cytoplasm and nucleus being enlarged. The more intense the growth processes had been, the more accentuated became the subsequent retrogressive changes in the cartilage cells of the transitional zone, as was indicated by the occurrence of pyknosis, karyorrhexis and karyolysis of such hypertrophic cartilage cells. Simultaneously, capillaries of the epiphyseal bone marrow advanced and corroded the bony border lamella which separates the cartilage of the joint from the epiphyseal bone marrow. These processes led to the appearance of minute arthropathic changes in the transitional zone of the articular cartilage, not noted at such an early age in normal mice irrespective of their strain. After preceding periods of injections extending over 3 to 4 months, the proliferation of the articular cartilage was less marked, but the retrogressive changes and particularly the replacement of the hypertrophic cartilage cells by bone had made progress. The bony border lamella, which had been thinned out at the earlier stages, was now greatly thickened.

When the injections were continued for periods of 10 months and longer, marked changes of the articular surface became noticeable. Active proliferation and hypertrophy of the cells of the sliding and transitional zones had set in in association with retrogressive changes (Fig. 8). The fibers of the cartilaginous matrix were teased apart and the hypertrophic cells were liquefied. Ulceration of the surface of the joint occurred, first near the insertions of the ligaments, and later spreading to other parts of the cartilaginous covering. Subsequently, processes of proliferation and retrogression affected also the ligaments and the capsule

of the joint, where an edematous swelling of the intercellular substance was seen. Furthermore, desquamation of amorphous masses of greatly hypertrophied cartilage cells was noted at the periphery of the joint. More or less severe arthritic lesions resulted from these changes.

C. Diaphysis. When anterior hypophyseal extract was injected for periods of from 1 week to 1 month, the periosteal connective tissue became softened and an increased mitotic proliferation of the periosteal cells was noted. A much larger number of spindle cells than usual were converted into round epithelioid cells, which likewise not infrequently underwent mitotic division. These cells were either transformed into bone cells and laid down osseous substance, or they formed giant cells and acted as osteoclasts. The cells lining the inner surface of the shaft showed changes similar to those in the periosteum, but to a lesser extent. The vascular canals were enlarged and congested and the compact bone, therefore, was somewhat less dense than normally. When the extract was administered for periods of from 3 to 6 months, the proliferation of the periosteal cells decreased and a dense collagenous and fibrillar connective tissue was formed. The shaft at this stage became harder, denser and slightly thicker than is normal. After continuation of the injections up to 1 year the conditions remained about stationary. In the second year of life, the vascular and lacunar absorption of bone was intensified and led to a thinning of the bone which was more pronounced than ordinarily.

The bone marrow was rich in cells during the first year of life; in the second year the tendency to form fatty marrow and fibrous tissue was increased, particularly in the epiphysis. But even in mice 18 months old which had been injected for 16 months, the replacement of the hemopoietic marrow by fat tissue and fibrous connective tissue was only partial and localized.

Strain Differences in the Response to the Stimulation by Anterior Pituitary Hormones

The degree of the reaction of the cartilage of the growth zones and joints after both implantation of hypophyseal glands and injections of extracts from cattle glands varied in different strains of mice. As a rule, mice belonging to strains C57 and CBA, which

have normally a slow rate of skeletal ageing, responded more readily and more strongly to the hormonal stimulation than did mice of strains A, D and C_3H, which normally exhibit a faster rate of skeletal ageing. In a C57 mouse 4 months old, which had received anterior pituitary transplants at the age of 2 months, the age changes in cartilage and bone were farther advanced than in a corresponding A mouse. Similarly, a C57 mouse 5 months old, which had been injected for 3 months, showed comparatively more degeneration and ossification of the growth zones and more osseous substance than an A mouse of the same age under corresponding experimental conditions. Strain differences were also noticeable at late experimental stages. Of two transplant-bearing mice 15 months old, one belonging to strain C57 and the other to strain C_3H, it was the C57 mouse in which the age changes of the skeleton were farther advanced. The same difference was found in two mice 17 months old of strains C57 and D which had received injections of anterior hypophyseal extract for 15 months, the age changes of the skeleton of the C57 mouse being ahead of those of the D mouse. Thus, as far as skeletal ageing is concerned, the order found in various strains under normal conditions is not maintained and may even become reversed under the influence of the anterior pituitary hormones.

Both the epiphyseal cartilage and the cartilage of the joint showed similar strain differences in the reaction towards hormonal stimulation. It was possible, in the joint, to distinguish three grades of changes: grade I was characterized by a slight but definite proliferation of the cartilage; grade II by a moderate degree of both proliferative and retrogressive changes; and grade III by marked proliferative and retrogressive changes which had produced pre-arthritic or marked arthritic lesions (Figs. 8 to 11).

The figures in Table III demonstrate that under the influence of the pituitary hormones the age changes in the joints are always intensified as compared with the normal condition. This intensification is indicated by a higher incidence, by a greater severity and by an earlier onset of these changes. Both pituitary extracts and implants call forth articular lesions, but the extracts exert a stronger effect than the transplants. Strain differences in the production of articular lesions become apparent if the figures for strains C57 and CBA are compared with those for strain D. In

TABLE III
The Distribution of the Degrees of Changes of the Articular Cartilage in Different Strains of Mice

	Age	Experiment	Number of mice	Negative	Grade of changes*		
					I	II	III
	mos.			*per cent*	*per cent*	*per cent*	*per cent*
Strains C57, CBA	1–5	Normal	15	100.0	0	0	0
	"	Transplant	10	56.7	33.3	10.0	0
	"	Extract	3	0	33.3	66.7	0
	6–18	Normal	33	42.4	30.3	18.2	9.1
	"	Transplant	13	15.3	30.8	30.8	23.1
	"	Extract	8	0	12.5	37.5	50.0
Strain A	1–5	Normal	9	100.0	0	0	0
	"	Transplant	14	42.8	21.4	35.8	0
	"	Extract	8	0	37.0	63.0	0
	6–14	Normal	10	10.0	50.0	40.0	0
	"	Transplant	32	9.4	25.0	40.6	25.0
Strain C3H	6–12	Normal	6	16.7	66.6	16.7	0
	"	Transplant	7	0	28.5	43.0	28.5
	"	Extract	7	0	28.5	43.0	28.5
Strain D	6–18	Normal	10	30.0	20.0	20.0	30.0
	"	Extract	7	14.3	14.3	28.6	42.8

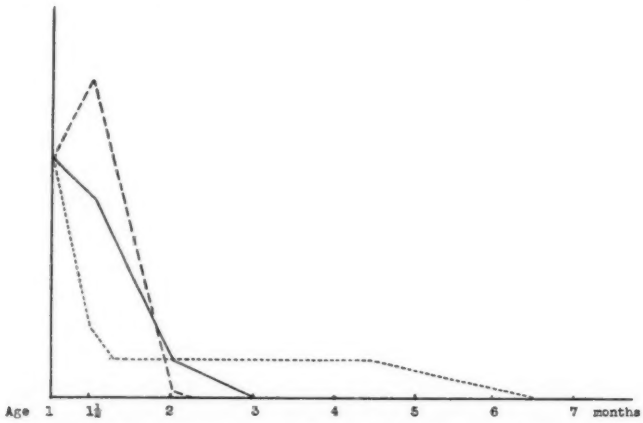
* The incidence of the changes taking place normally and under experimental conditions is given in percentages of the total number of mice used in each group.

both groups the experiments were continued until the animals had reached the age of 18 months. It is therefore possible to compare the figures obtained in these groups. In strains C57 and CBA, the normal incidence of the articular changes during the period from 6 months on is, for grades II and III, lower than in strain D. Under the influence of pituitary extract, however, strains C57 and CBA show a more marked increase in the articular changes than does strain D. No negative cases are recorded; cases with lesions of grade I show a diminution of 17.8 per cent, whereas those with lesions of grades II and III show an increase of 19.3 and 40.9 per cent respectively. Conversely, in strain D, in which the normal incidence of articular changes is much higher than in strains C57 and CBA, the increase due to the administration of the pituitary extract is relatively smaller than in strains C57 and CBA. The number of negative cases diminishes by 15.7 per cent; cases with lesions of grade I show a decrease of 5.7 per cent, and cases with lesions of grades II and III show an increase of only 8.6 and 12.8 per cent, respectively, as compared with the normal values for this strain.

Thus the course of the articular changes under the influence of stimulation by pituitary hormones is, to a certain extent, influenced by the strain to which the animal belongs. Mice exhibiting normally little tendency to develop articular age changes are more severely affected than those having a greater tendency to develop these changes spontaneously. The reaction of the articular cartilage, therefore, resembles that which takes place in the epiphyseal cartilage under the influence of the pituitary hormones.

Comparison of the Effects of Syngenesiotransplants and Extracts of Bovine Anterior Hypophysis on Cartilage and Bone

The following text-figures present the course of the proliferation of the cartilage of the growth zones, the degenerative changes in this cartilage and the formation and the absorption of bony



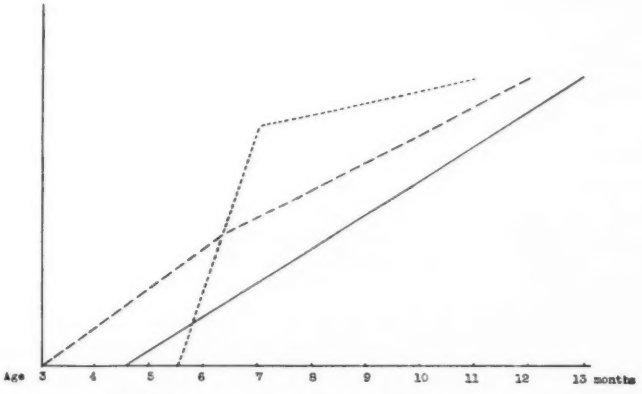
——— Normal,. . . . . Ant. pit. implants,------Ant. pit. extract.

Text-Fig. 1. The proliferation of the cartilage of the growth zones.

tissue in this area and in the shafts of the long bones under normal and experimental conditions.

Text-Figure 1. The Proliferation of the Cartilage of the Growth Zones. In normal mice the degree of proliferation of the epiphyseal cartilage decreases gradually from the age of 1 month to the

age of 4 months, after which time it practically ceases. During the first 2 weeks subsequent to pituitary transplantation the proliferation of the epiphyseal cartilage decreases much more rapidly than under normal conditions. This causes the epiphyseal cartilage to have, at this time, the appearance of cartilage 1 to 2 months older than its actual age. Following this period of rapid decline the proliferation continues at this decreased level up to the age of 5 to 6 months, while normally proliferation ceases entirely at about the age of 3½ to 4 months. Therefore, the epiphyseal cartilage looked younger at this stage than is normal. At the age of 7 months the proliferation of the cartilage in the animals bearing transplants had ceased. In mice receiving injections of pituitary extract, the proliferation of the epiphyseal cartilage was greatly increased during the first 2 weeks, after which period it declined very rapidly, but it was still above the normal level 4 weeks after the beginning of the injections. It then sank below normal and came to a standstill at about the



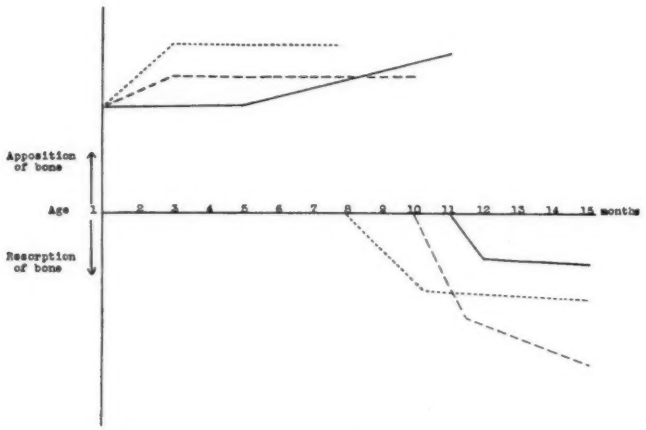
——— Normal, ... Ant. pit. implants, – – – – – – Ant. pit. extract.

TEXT-FIG. 2. The degenerative changes in the cartilage of the growth zones.

age of 3 months. At the age of 3 months the epiphyseal cartilage had an appearance comparable to that of normal mice at the age of almost 4 months.

Text-Figure 2. The Degenerative Changes in the Cartilage of the Growth Zones. In normal mice the retrogressive changes ap-

peared at the age of about 4 months, or slightly later, and progressed steadily. Under the influence of the transplants the retrogressive processes began about 1 month later than in untreated animals, but they proceeded more rapidly and reached their maximum at the age of 10 to 11 months, which is earlier than normally. After administration of pituitary extract, the retrogressive changes were seen approximately 1 to 1½ months earlier than usual. During the first year, they made gradual progress and were always farther advanced than in normal animals.



——— Normal, ... Ant. pit. implants, – – – – – – Ant. pit. extract.

TEXT-FIG. 3. The formation and absorption of bony tissue.

Text-Figure 3. The Formation and Absorption of Bony Tissue. In normal mice there was a fairly constant apposition of bone up to the end of the first year of life. In transplant-bearing mice, bone formation was increased over the normal during the first 8 months of life. This, in association with the increased degeneration shown in Text-Figure 2, led to a premature closure of the epiphyseal disk. At the age of 8 months, solution processes began to predominate; changes which were earlier and more intense than ordinarily. Subsequent to the administration of anterior pituitary extract, bone formation was increased over the normal degree, but less pronounced than after transplantation. Resorp-

tive processes set in at an earlier date than in untreated animals, but later than after syngenesiotransplantation of pituitary glands.

We may then conclude that both syngenesiotransplants and extracts of the bovine anterior pituitary accelerate the skeletal ageing of mice. However, there are some differences in the action of these two substances. The extracts call forth an initial brief rise in the proliferation of the epiphyseal cartilage which is followed by an accelerated retrogression. The primary effect of transplants is a decrease of the growth of cartilage, associated with a prolongation of the growth period and followed by an even greater acceleration of the degenerative processes. In addition, there is a further difference in the degree of the changes induced by transplants on the one hand, and pituitary extract on the other. More bony substance is found after transplantation than after administration of the extract, and, at later stages, resorptive processes are more marked in injected animals than in transplant-bearing mice.

Discussion

Syngenesiotransplants of the anterior lobe of the hypophysis of mice survive and, in the mammary gland, increase the incidence of cancer (Loeb and Kirtz [4]). But in bone and cartilage, syngenesiotransplants of the anterior pituitary gland, instead of increasing the proliferation of cartilage, cause at first a definite inhibition of growth which lasts for some time. Subsequently, this deficiency of growth is balanced by a prolongation of the period during which proliferation takes place. Suppression of growth was likewise observed in urodele larvae which had received homeotransplants of embryonal hypophysis (Blount [6,7]), and no growth promotion was obtained in similar experiments on anuran larvae by Smith,[8] Allen,[9-11] and Burns.[12] On the other hand, increased lengthwise growth of the long bones of rabbits subsequent to homeotransplantation of whole pituitary glands has been reported by Pereira.[13] No changes were noted in the epiphyseal disks of these rabbits, but the increase in growth was believed to have been due to a prolongation of the growth period. However, an extension of the growth period can be diagnosed only when there is an abnormal persistence of growth of the cartilage, and it should be possible to recognize such a condition

by microscopical comparison of the epiphyseal cartilage of the experimental animals with that of normal animals of the same age.

In contradistinction to the anterior pituitary transplants, extract of the bovine anterior pituitary exerts, in the beginning, a temporary stimulation on the proliferation of cartilage. Subsequently, the growth period is shortened and thus compensation for the temporary overgrowth takes place and gigantism does not occur. Conditions in mice seem to resemble, therefore, those observed in guinea pigs [2] in which, after prolonged injections of anterior pituitary extract, there was likewise, following the transitory growth stimulation, a readjustment which prevented the occurrence of gigantism.

The difference in the response of the epiphyseal cartilage to the administration of the anterior pituitary hormone in diverse strains of mice may explain some variations which we [2] observed in the reaction of the cartilage in guinea pigs. Under apparently identical experimental conditions, proliferation of cartilage predominated over ossification in some animals, while the opposite condition was noted in others during the greater part of the experimental period. There are some indications that in this species also, at later stages, an adjustment takes place which restores the balance between the processes of proliferation and ossification. In the present experiments in mice the time of observation was much longer than in our earlier investigations in guinea pigs, the former being about 1½ years as compared with 6 months in guinea pigs. Furthermore, 1½ years in the life of a mouse corresponds to a much longer period in the life of a guinea pig. In view of the fact that mice of some strains react with a more rapid proliferation which is followed by a more marked degeneration and ossification, it may be suggested that in the guinea pig, also, similar strain differences may exist and that such differences may be responsible for the varying response of the cartilage to stimulation by the pituitary hormone in different individuals.

The microscopic observations recorded by us are in agreement with the statements by Allen [9-11] and Smith [8] that in animals which have a sufficient supply of anterior pituitary hormone, an additional amount of this hormone cannot produce a permanent in-

crease of growth beyond the normal degree. The primary effects which we have observed; namely, a diminution of the proliferative rate of the cartilage in animals with transplants and a primary stimulation in the case of extract, are temporary and are subsequently neutralized by a balancing mechanism.

Transplants and extracts of anterior hypophysis exert in addition, however, an effect on the skeleton which is permanent and which is not neutralized. This common effect is an acceleration of the normal processes of ageing which occurs simultaneously in various parts of the skeleton. In the growth zones of the long bones the epiphyseo-diaphyseal union is accelerated; in the shaft, senile absorption sets in prematurely; and in the joints, arthritic lesions are increased or prematurely initiated. In other animals anterior hypophyseal preparations have also been shown to cause an acceleration of development and ageing. Swingle [14] and Allen [9-11] observed in frog larvae, and Clements and Howes [15] in axolotl, an acceleration of metamorphosis under the influence of anterior pituitary hormone. Downs [16] obtained a premature shedding of the deciduous teeth and precocious eruption of the permanent teeth in dogs subsequent to the administration of anterior pituitary hormone.

The question arises as to whether the anterior pituitary hormone induces the skeletal changes directly or by the intermediation of other endocrines. In mice, pituitary transplants do not exhibit a very great thyrotropic activity, but they exert a marked gonadotropic effect (Loeb and Kirtz [4]). Since, under the influence of the pituitary transplants, the output of estrogen is perhaps accelerated or increased, the changes in the long bones might possibly be due to this increase in the amount of estrogen. Previously we [5] have shown that in normal mice, skeletal ageing proceeds more rapidly in strains with high mammary cancer incidence, and in breeding females. Furthermore, a similarity exists in the microscopic changes taking place in cartilage and bone of mice bearing transplants and in guinea pigs treated with estrogen.[17,18] In both cases the early effect is an inhibition of proliferation of cartilage. This early reaction is followed by a second, characterized by a relative increase in proliferation, and by a third, characterized by an intense degeneration and ossification of cartilage.

It is therefore possible that the transplants of anterior hypophysis act in two ways on cartilage and bone: they may act directly; or they may induce a greater production of estrogen and secondarily affect bone and cartilage by way of this hormone. Investigations in spayed mice are being conducted to determine whether an estrogen plays any rôle in the effect of pituitary transplants on the skeleton.

As to the effect of the anterior pituitary extract on cartilage and bone, this is present in thyroidectomized as well as in ovariectomized guinea pigs,[19,20] and in thyroidectomized salamanders (Richardson [21,22]). It appears probable, therefore, that also in mice the pituitary extract may act directly on the skeletal tissues.

Summary

Anterior hypophyseal hormone exerts in mice (1) primary effects of a transitory nature on the growth of the epiphyseal cartilage and (2) permanent effects on cartilage and bone. The primary effects differ depending upon whether they are due to syngenesiotransplants or to intraperitoneally injected bovine hypophyseal extract. Under the influence of the former, the proliferation of the epiphyseal cartilage is at first inhibited, and this inhibition is followed by a prolongation of the period during which growth takes place. Bovine anterior pituitary extract causes at first a stimulation of proliferation of the epiphyseal cartilage, which is followed by a sharp decline and by a diminution in the duration of the growth period. The later and permanent effect of both syngenesiotransplants and bovine anterior hypophyseal extract is an acceleration of skeletal ageing. These changes lead to an accelerated epiphyseo-diaphyseal union, a premature absorption of the bony tissue of the shaft of the long bones, and an increase in the incidence and degree of arthropathic lesions. Strain differences determine, to a certain degree, the response of the skeletal tissues to the anterior hypophyseal hormones; mice of strains C_{57} and CBA reacting more intensely than those of strains A, C_3 H and D.

Note: We wish to express our indebtedness to Leo Loeb for his advice in this study and to S. J. Hayward for the photomicrographs.

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DESCRIPTION OF PLATES

PLATE 42

FIG. 1. Section through the growth zone of the upper tibia of a normal virgin mouse of strain A, 8 weeks old. Epiphyseal line is wide and open. In the subepiphyseal zone, which is well vascularized, there is formation of trabeculae. × 150.

FIG. 2. Section through the growth zone of the upper tibia of a virgin mouse of strain A, which, beginning at the age of 7 weeks, had received injections of bovine anterior pituitary extract for 1 week. Many columnar cartilage cells are vesicular and the number of large hypertrophic cartilage cells is decreased. In the subepiphyseal zone the trabeculae are more numerous, longer and thicker than in Figure 1, their base being close to the zone of hypertrophic cartilage. × 150.

FIG. 3. Section through the growth zone of the upper tibia of a virgin A mouse which, at the age of 7½ weeks, had received four syngenesiotransplants of the anterior lobe of the hypophysis and which had been sacrificed 1 week subsequent to transplantation. The growth zone is narrowed and partly calcified. The cartilage cells are decreased in size. The subepiphyseal zone is poorly vascularized. Thick and dense trabeculae reach up to the epiphyseal cartilage more closely than in Figures 1 and 2. × 150.

1

2

3

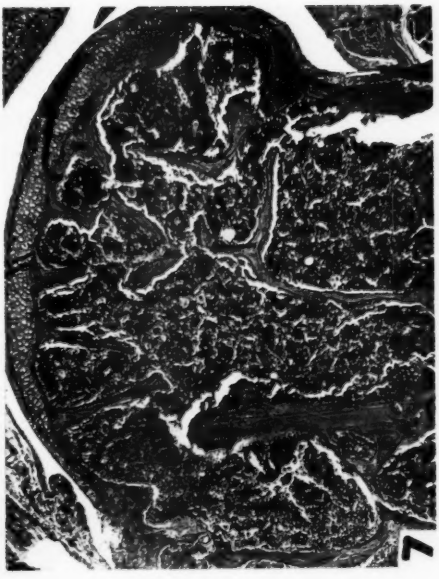
PLATE 43

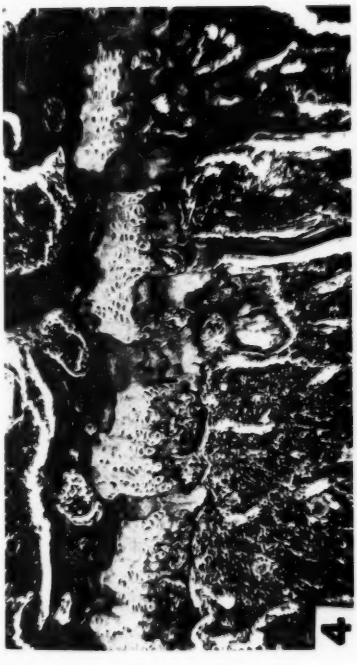
FIG. 4. Section through the growth zone of the upper tibia of a virgin C57 mouse which, from the age of 2 months on, had received injections of bovine anterior pituitary extract for a period of 3 months. The cartilaginous ground substance is increased and hyalinized and numerous thick plugs have taken the place of disintegrated cartilage cell rows. There is formation of a transverse bony plate underneath the epiphyseal cartilage. × 150.

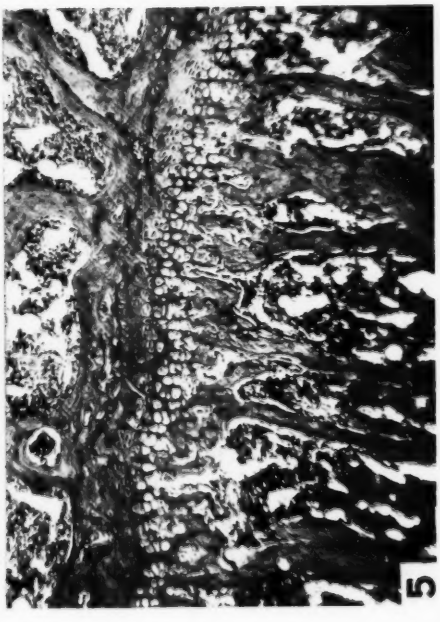
FIG. 5. Section through the growth zone of the upper tibia of a virgin A mouse 5 months old which had received four syngenesiotransplants at the age of 2 months. There is no degeneration of the epiphyseal cartilage visible; the conditions in the epiphyseal cartilage are similar to those shown in Figure 3. × 150.

FIG. 6. Section through the growth zone of the upper tibia of a virgin A mouse 10½ months old which had received four syngenesiotransplants at the age of 1½ months. The epiphyseal cartilage is almost completely ossified and only scanty remnants of hyalinized cartilage are left. The cartilage is enclosed on both sides by a thick bony plate. In the center of the disk advancing bone marrow is seen. × 150.

FIG. 7. Section through the lower femur of a male C57 mouse 17½ months old which, from the age of 2½ months, had been injected with bovine anterior pituitary extract. The epiphyseal disk has been resorbed and complete epiphyseo-diaphyseal union has taken place. × 100.







Silberberg and Silberberg Effects of Hypophysis on Mice

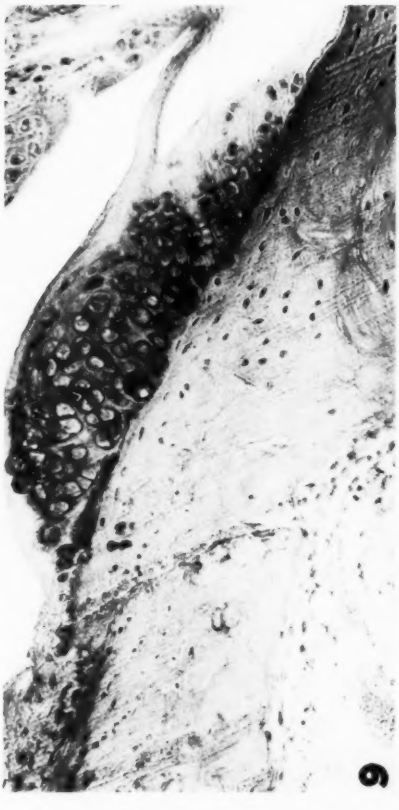
PLATE 44

FIG. 8. Section through the articular surface of the knee joint of a male D mouse 12½ months old which had been injected with bovine anterior pituitary extract for 10 months. Hyperplasia and hypertrophy of the articular cartilage are present. (Changes: grade I.) × 220.

FIG. 9. Section through the articular surface of the knee joint of a virgin A mouse 11 months old which had received four syngenesiotransplants of anterior pituitary at the age of 4 weeks. Moderate hyperplasia and hypertrophic growth of the articular cartilage are present near the insertion of a ligament. In some places, cells show degenerative changes; the cartilaginous covering is in process of solution. (Changes: grade II.) × 220.

FIG. 10. Section through the articular surface of the knee joint of a virgin A mouse 14 months old which had received five syngenesiotransplants of anterior pituitary at the age of 3 months. There is an outgrowth of hypertrophic cartilage near the insertion of the capsule of the joint. (Changes: grade III.) × 220.

FIG. 11. Section through the articular surface of the knee joint of a virgin A mouse 12½ months old which had received four syngenesiotransplants of the anterior pituitary at the age of 1½ months. Marked proliferation and degeneration of the articular cartilage are present. Ligaments show an edematous swelling and degeneration of cells. (Changes: grade III.) × 220.



Silberberg and Silberberg

Effects of Hypophysis on Mice

DEGENERATE VERSUS MULTIPOLAR NEURONS IN SENSORY GANGLIA *

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Evidence has been presented for (Horton-Smith;[1] Kuré *et al.*;[2,3,4] Okelberry;[5,6] and Kahr and Sheehan[7]) and against (Duncan and Crocker;[8] and Hinsey[9,10]) the existence of spinal parasympathetic nerve fibers. Such attempts to challenge the Bell-Magendie law have been based on experimental degeneration of dorsal root fibers following root resection and on morphological studies of spinal ganglia under normal and experimental conditions.

Westbrook and Tower[11] have recently thrown new light upon the problem in their description of extraneous fibers which grow into the dorsal root following root resection and ganglionectomy. A detailed discussion of the recent literature can be found in their report.

The existence of parasympathetic fibers in the dorsal roots of spinal nerves would necessitate the presence of efferent (multipolar) neurons in either the spinal ganglia or within the spinal cord. Kuré, Saégusa, Kawaguchi and Shiraishi[3] have described such cells of intraspinal origin as lying in the ventral portion of the dorsal horn, while Kiss[12] has described small, dark multipolar neurons in the spinal ganglia following prolonged osmification. Fisher and Ranson[13] concluded that the stellate appearances of the dark cells described by Kiss were the result of cell injury and shrinkage.

In view of the above conflicting reports, a more extensive investigation with appropriate silver methods seemed desirable. By studying the finer ramifications of neuron processes in normal, senile and pathologic sensory ganglia one might expect to observe true multipolar neurons, should they exist.

* Received for publication July 30, 1940.

Material and Methods

Normal spinal and gasserian (semilunar) ganglia of 7 white leghorn chickens, 5 white rats, 10 adult cats and 3 rhesus monkeys were perfused with a neutral 4 per cent solution of formaldehyde and dissected for study. In addition, 57 human gasserian ganglia and 7 spinal ganglia from cervical, thoracic and lumbar levels were obtained at autopsy. The sources of the human specimens ranged from the newborn to 81 years of age and specimens were selected regardless of sex, clinical symptoms or autopsy findings. Celiac and lumbar sympathetic ganglia were obtained from 8 human bodies for purposes of comparison.

Fixation was in ammoniated absolute alcohol, 10 per cent alcoholic chloral hydrate, or neutral 4 per cent solution of formaldehyde. Following fixation the tissues were carried through the Ranson pyridine-silver, and the Cajal silver impregnation methods. Sections of silver preparations were cut in a craniocaudal plane at 12 μ. The formaldehyde-fixed specimens were cut at 8, 10 and 12 μ and alternate sections stained by the Bodian protargol and cresyl-violet technics. Serial sections of each specimen were examined, using an oil immersion lens as needed.

Observations

Earlier investigations concerning structural changes in sensory neurons concomitant with senility in the chicken and man have been reported by me.[14,15] During the course of these studies the atypical and degenerate neurons have constantly aroused interest, not only because of their numerous and complexly ramifying processes, but also because of their marked resemblance to true multipolar neurons. Such similarity is demonstrated by sensory cells possessing supernumerary processes, commonly designated as "frayed cells of Cajal." These alone are considered in this investigation.

It would not seem possible, to one examining the sensory ganglia for the first time, that a normal unipolar neuron (Fig. 1) could undergo extensive transfiguration and thereby be confused with the structure of a multipolar neuron (Fig. 2). Yet, if one compares the appearances of the degenerate sensory cells in Figures 3, 6, 9, 12, 15 and 18 with those of sympathetic neurons in

Figures 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19 and 20, differentiation between the two cell types becomes progressively more difficult.

There appears to be no limitation on the number of such accessory processes of a unipolar cell, or on the complexity which each process may attain. With the addition of each new process, originating from either cell soma or undivided stem (axon), the sensory neuron more closely resembles the multipolar type.

In Figure 3 the accessory processes arise only from the axonic stump, and sensory neurons with this structure bear a likeness to the truncated multipolar cells of the sympathetic ganglia (Figs. 4 and 5). Sensory neurons demonstrating only a few short, thick radicles (Fig. 6) are similar to efferent neurons which possess a like number of processes (Figs. 7 and 8).

The degenerate unipolar cells in Figures 9, 12 and 15 are very common in senile and pathologic sensory ganglia of man. Due to the increase of processes and collateral branching, such neurons are easily confused with diverse sympathetic cells of closely parallel structure (Figs. 10, 11, 13, 14, 16 and 17).

Excessive proliferation of cytoplasmic outgrowths and cell excrescences results in the bizarre structure shown in Figure 18. The dichotomous branchings and profuse ramifications on these sensory neurons might readily lead one to assume that he was observing a motor neuron. The distinction is at times quite difficult in view of the similar structure and branching of the multipolar cells (Fig. 19). In addition, the so-called "comet-cells" of the sympathetic nervous system (Fig. 20) manifest a wealth of short branching processes, whereby they too simulate degenerate sensory components like those shown in Figure 18.

The features distinguishing between degenerate sensory and true multipolar neurons are shown in the accompanying figures. The short accessory processes of the sensory cell usually terminate within the limits of the surrounding capsule. The frequency of branching, the varicosities, and the terminal club-shaped expansions along the processes provide additional distinguishing criteria. Likewise, the abortive cell outgrowths stain more darkly with the usual silver impregnation methods than does the normal undivided stem of the sensory neuron.

In contrast to these features, the more lightly stained processes of the efferent cells pass through the confines of the cell capsule

as definite axon or dendrite radicles. They possess few or no club-shaped outgrowths or reticulated expansions. Branching of the true dendrites is more uniform and equal.

In sympathetic ganglia from older specimens, both animal and human, one frequently observes a definite thickening or hypertrophy of neuronic processes (Figs. 5 and 14). In other instances all of the dendritic processes do not penetrate the capsule (Figs. 11, 13, 14, 17, 19 and 20). As a result of these inconsistencies, the contrasting features as listed may be less clearly defined.

Serial examination of all the animal and human specimens revealed no cell which could be classified morphologically as a true multipolar neuron.

Distribution of Degenerate Neurons. Spinal and gasserian ganglia of the chicken, rat, cat, monkey and man possess the described degenerate neurons in variable numbers. Though relatively few in the spinal ganglia of the lower animals, they are more frequent in the animal gasserian and human spinal ganglia. Festooned sensory cells are abundant in human gasserian ganglia from patients of all ages. Their occurrence is particularly frequent following death from diabetes, chronic alcoholism, barbital poisoning and prolonged infectious diseases.

Comment

Embryologically, it would be possible to account for the presence of multipolar neurons in the sensory ganglia since the primordia of the spinal ganglia lie along the course of migrating neuroblasts destined for the sympathetic ganglia. Decision whether such neuroblasts are derivatives of the neural crest, neural tube, or both, awaits further investigation. In any case the opportunity presents itself at this early stage for migrating neuroblasts destined to become efferent neurons to be retained within the definitive spinal ganglia. Subsequent differentiation of the retained neuroblasts into multipolar neurons, which were later reached by preganglionic fibers, might then offer a plausible explanation for the presence of so-called spinal parasympathetic fibers in the dorsal root of spinal nerves.

In a similar way the gasserian ganglion might be expected to manifest such retained neuroblasts, since the primordia of the ciliary, submaxillary, sublingual and sphenopalatine ganglia are

known to arise in close association with branches of the trigeminal nerve (Streeter,[16] Stewart [17]).

This assumption or retention theory is not wholly free from justified criticism. However, it affords a legitimate explanation for, and understanding of the significance of, multipolar neurons should they be found to exist in sensory ganglia. From the study of serial silver preparations of spinal and gasserian ganglia designed to demonstrate such cells, no morphological evidence could be found to substantiate their existence. In the absence of efferent neurons within the sensory ganglia, the reality of preganglionic efferent fibers in the dorsal spinal root is questionable.

The presence of many degenerate sensory neurons, designated by Cajal [18] as "frayed cells" in view of their ragged and cogwheel appearance, might readily lead to confusion of neuron cell types. In addition, terminology becomes confusing if investigators classify ganglion cells with supernumerary processes as multipolar. Such cells are multipolar only in the sense that they possess more than two processes and in these instances the term cannot be applied with the intention of denoting a motor or efferent physiological nature.

It should be pointed out that no technic can be utilized in determining the multipolarity of a ganglion cell unless it is capable of staining the individual processes and demonstrating their capsular and extracapsular relationship. The classification of neuron cell types following the osmification method described by Kiss [12] has been found unreliable, as was pointed out by Fisher and Ranson [13] and more recently by Levi and Meyer.[19]

Neurons herein described as atypical must be looked upon as degenerate cells. Due to clumping of the cytoplasmic tigroid bodies in senile sensory cells (Truex [15]), the cresyl-violet and Nissl technics cannot be relied upon to distinguish adequately between all sensory and motor neurons.

Summary

Serial silver preparations of the spinal and gasserian ganglia of the chicken, white rat, cat, monkey and man fail to demonstrate the presence of multipolar neurons. No morphological evidence has been presented to date which conclusively substantiates the existence of such neurons of parasympathetic function within

the spinal or gasserian ganglion. The reality of preganglionic efferent fibers terminating in spinal ganglia remains questionable.

Degenerate sensory neurons often simulate multipolar cells. These two cell types may be distinguished from each other by the length, branching, varicosities and staining of their respective processes.

Osmic acid and Nissl technics are inadequate methods with which to classify nerve cell types, due to structural variations of sensory neurons in senile and pathologic ganglia.

Recognition of cell types and variation in the appearances of degenerate neurons is essential for correct interpretation of sensory ganglionic structure.

NOTE: The author wishes to express his appreciation to S. R. Detwiler and A. T. Rasmussen for their coöperation and helpful criticism throughout this investigation. He is indebted to the Departments of Pathology of the University of Minnesota and of the College of Physicians and Surgeons of Columbia University for aid in obtaining fresh specimens and clinical data. The author expresses his thanks to Julius Bloom for valuable assistance in the preparation of photomicrographs.

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DESCRIPTION OF PLATES

PLATE 45

All figures are untouched photomicrographs from silver preparations of human material.

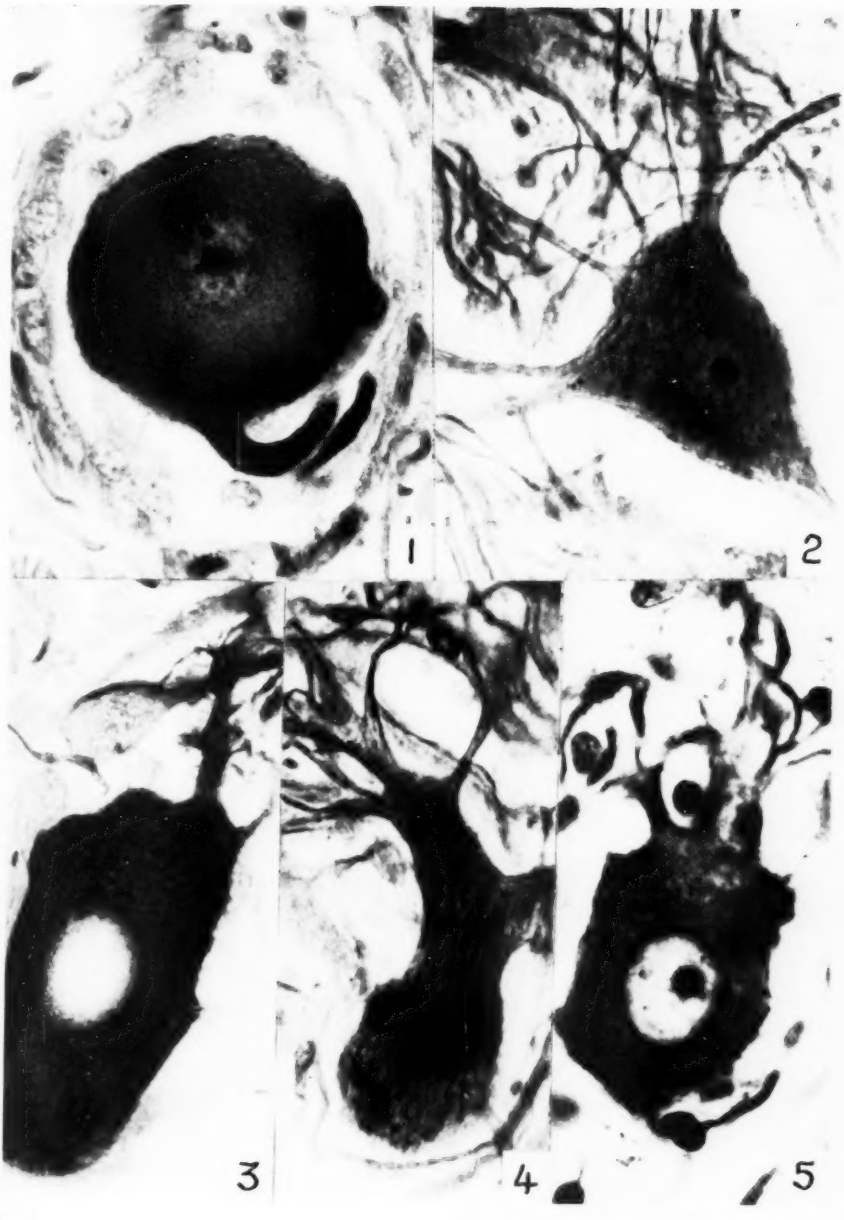
FIG. 1. Normal unipolar neuron with capsule and process (axon). Gasserian ganglion. Male, 2½ years old. Cajal's technic. × 1000.

FIG. 2. Typical multipolar neuron with processes. Celiac ganglion. Female, 42 years old. Cajal's technic. × 1000.

FIG. 3. Degenerate unipolar neuron showing short extraneous outgrowths and a clublike expansion arising from its single process. Gasserian ganglion. Female, 66 years old. Cajal's technic. × 1000.

FIG. 4. Truncated multipolar neuron. Celiac ganglion. Female, 42 years old. Cajal's technic. × 1000.

FIG. 5. Truncated multipolar neuron with moderate hypertrophy of processes. Third lumbar sympathetic ganglion. Male, 59 years old. Bodian's technic. × 1000.



Truex

Degenerate Versus Multipolar Neurons

PLATE 46

FIG. 6. Degenerate unipolar neuron possessing three thick intracapsular processes. Gasserian ganglion. Male, 65 years old. Cajal's technic. × 800.

FIG. 7. Multipolar neuron with similar number of processes as cell in Figure 6. Celiac ganglion. Female, 42 years old. Cajal's technic. × 800.

FIG. 8. Multipolar neuron with three processes. Third lumbar sympathetic ganglion. Male, 34 years old. Bodian's technic. × 800.

FIG. 9. Degenerate sensory neuron with many short accessory processes arising from cell soma. Gasserian ganglion. Female, 66 years old. Cajal's technic. × 800.

FIG. 10. Multipolar cell with processes arising from cell soma. Celiac ganglion. Female, 42 years old. Cajal's technic. × 800.

FIG. 11. Multipolar neuron with short dendritic processes. Note that all processes do not penetrate the investing capsule. Third lumbar sympathetic ganglion. Male, 59 years old. Bodian's technic. × 800.

FIG. 12. Degenerate sensory neuron with supernumerary processes arising from entire circumference of cell. Note variation in thickness and terminal expansions of these radicles. Gasserian ganglion. Male, 65 years old. Cajal's technic. × 800.

FIG. 13. Multipolar neuron with branching processes. Third lumbar sympathetic ganglion. Male, 77 years old. Cajal's technic. × 800.

FIG. 14. Multipolar neuron with short branching processes. Note similarity to degenerate neuron in Figure 12. Third lumbar sympathetic ganglion. Male, 59 years old. Bodian's technic. × 800.

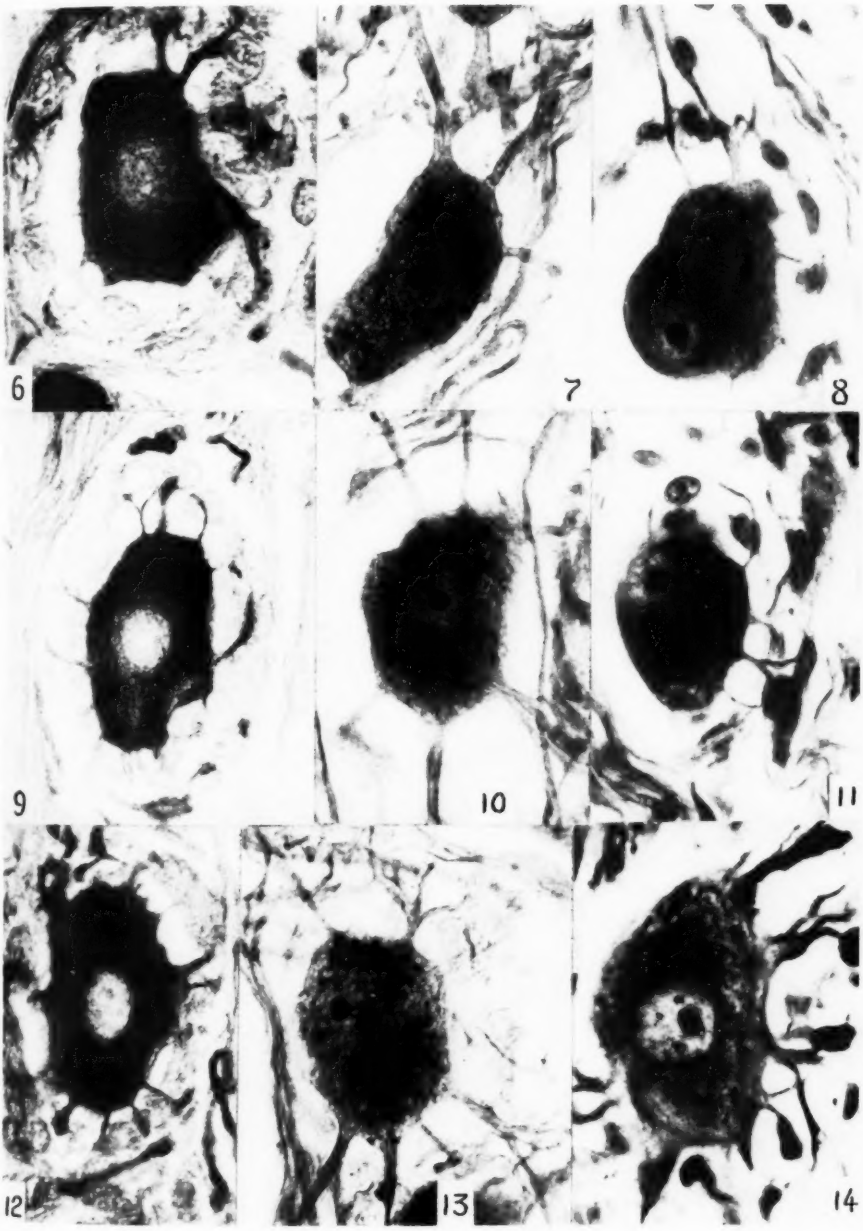

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PLATE 47

FIG. 15. Degenerate unipolar neuron demonstrating short branching processes. Gasserian ganglion. Male, 65 years old. Cajal's technic. × 800.

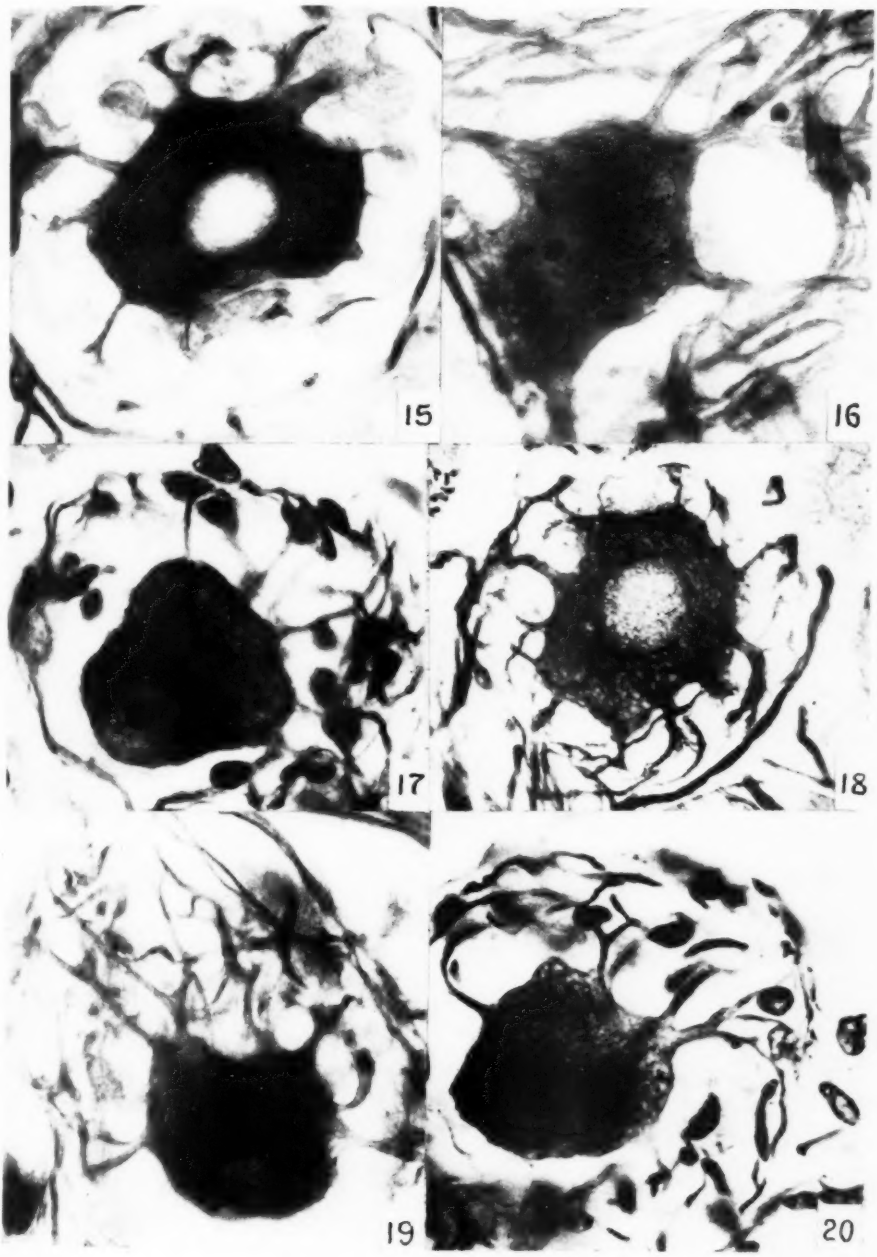
FIG. 16. Multipolar neuron with process formation similar to the degenerate cell in Figure 15. Female, 42 years old. Cajal's technic. × 800.

FIG. 17. Multipolar neuron with short intracapsular processes. Third lumbar sympathetic ganglion. Male, 59 years old. Bodian's technic. × 800.

FIG. 18. Degenerate unipolar neuron with extreme branching of processes. Note the wealth of primary, secondary and tertiary rami on this cell. Gasserian ganglion. Female, 66 years old. Cajal's technic. × 800.

FIG. 19. Multipolar neuron with process formation similar to the degenerate sensory cell shown in Figure 18. Third lumbar sympathetic ganglion. Male, 77 years old. Cajal's technic. × 800.

FIG. 20. Multipolar neuron. Commonly designated as "comet-cell" of sympathetic nervous system. Note intracapsular branching of processes. Celiac ganglion. Male, 55 years old. Bodian's technic. × 800.



Truex

Degenerate Versus Multipolar Neurons

UNIV. OF MICH.

STUDIES ON LEUKOCYTOSIS *

I. Hourly Leukocyte Variations of Normal Rabbits

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Few studies have been reported of the variations of individual rabbits whose leukocytes have been counted at short intervals during the day. The purpose of this report is to present results of total and differential leukocyte examinations of 14 normal rabbits, studied at hourly intervals.

In the report by Sabin, Cunningham, Doan and Kindwall [1] of "the normal rhythm of the white blood cells" in human subjects, brief mention is made that experiments on the blood of rabbits, similar to the reported experiments on humans, showed "the same general rhythms." They published one chart of one rabbit, studied at consecutive 30 minute intervals from 11 a.m. to 1:15 p.m. This chart shows considerable variation in total leukocyte counts and numbers of polymorphonuclear neutrophilic leukocytes (amphophils) and lymphocytes. Bushnell and Bangs [2] tabulated the total and differential leukocyte counts of one rabbit, examined at consecutive 45 to 60 minute intervals during the day on five occasions over a period of 43 days. On each day no determinations were made during a 2-hour interval around noon. Under these conditions, considerable daily variation occurred in the leukocyte counts of this rabbit. Cheng [3] presented the total leukocyte counts of two rabbits, studied at consecutive 15 to 30 minute intervals. One rabbit was examined during the afternoon of one day and again during the morning of the following day. The other rabbit was studied during the afternoon of one day and again, 2 days later, from late morning through the afternoon. Cheng stated that the total counts of both rabbits tended to show

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similar variations, consisting of two rises, one at 10 a.m. to 12 noon, and the other at 3 to 4 p.m. There were also sudden variations of about 2500 leukocytes at short intervals.

In these reports, no mention is made of the number of 1 sq. mm. unit areas which were examined in determining the total leukocyte counts of each rabbit. Sabin and co-workers [1] did not state the number of leukocytes which they studied in making the differential examinations of their rabbit. Bushnell and Bangs [2] counted and classified approximately 200 leukocytes in making their differential determinations.

Despite the scarcity of reports of daily leukocyte variations of individual rabbits, a voluminous literature has accumulated concerning other aspects of leukocyte variations of rabbits. This literature has been summarized by Cheng [3] and Garrey and Bryan.[4] An analysis of errors in leukocyte counting has been presented by Bryan, Chastain and Garrey.[5] Anyone working with variations in leukocyte counts is impressed with the numerous possible sources of error in the determinations, involving all procedures from selection of subjects to the actual counting of the leukocytes.

A large number of cells should be counted in making total and differential leukocyte determinations if significant results are to be obtained. The importance of counting enough cells in making total leukocyte counts is evident from studies of leukocyte variations in normal human subjects by Sabin and co-workers [1] and Ponder, Saslow and Schweizer.[6] Sabin *et al.*, counting the cells on "the same side of a counting chamber" in five of six total leukocyte determinations, described hourly rhythmical leukocyte variations. Ponder and co-workers obtained similar apparent hourly rhythms when the cells on "one side of one counting chamber" were counted, but noted that these rhythms tended to disappear and much less fluctuation in counts occurred when the cells on "both sides of 2 counting chambers" were counted. It seems advisable to record the number of cells enumerated in making total leukocyte counts in terms of a standard, such as "unit areas of one square millimeter each," recognizing that one side of a certified counting chamber contains nine such unit areas.[5]

After every attempt has been made to eliminate possible sources of error in the determinations, irregular variations may

still occur in leukocyte counts of rabbits made at short intervals during the day.[4,7] Observations of human leukocyte variations indicate that fairly frequently the total leukocyte count increases moderately in the afternoon, so that the average of the afternoon counts may be higher than the average of the morning counts.[1,6,8] In many instances this afternoon increase in human subjects is apparently due mainly to an absolute increase in the number of polymorphonuclear neutrophilic leukocytes.[1,8,9]

The following observations of hourly leukocyte variations of 14 normal rabbits are presented. These studies were made in connection with experiments on leukocytosis to be reported subsequently.

Procedure

Fourteen adult New Zealand white and mixed rabbits, weighing approximately 2.5 to 3.2 Kg. each, were studied during the period from May 24, 1939 to January 23, 1940. Each animal was kept in the same individual cage throughout this period and for several months prior to the day of counting. During this time the rabbits were removed from their cages at least once daily and appeared adjusted to handling. The temperature of the cages and counting rooms was maintained at approximately 20 to 25° C. One animal (B) was counted for two series of observations. No differences were noted between the counts of males and females, and both sexes were used. Rabbits with ear canker, snuffles, or any other abnormality, including abnormal eating or activity, were discarded. As the incidence of coccidia infestation appears worthy of consideration in reports dealing with leukocyte variations of the rabbit,[10] mention should be made that the incidence of coccidiosis has been low in autopsied animals of similar breeds kept under identical conditions.

Food was placed in each rabbit cage once daily at approximately the same time (9 to 11 a.m.). The animals ate slowly over a period of 3 to 4 hours. The diet consisted of a complete ration chow, carrots and water. The rabbit chow was guaranteed by the manufacturer to have the following analysis: crude protein not less than 13.5 per cent, crude fat not less than 2.5 per cent, carbohydrate in crude fiber not less than 16 per cent, and carbohydrate in nitrogen-free extract not less than 47 per cent. It was

guaranteed to contain the following ingredients: wheat germ, soy bean oil meal, cut alfalfa, crushed oats, crushed barley, wheat middlings (standard), corn germ meal, crushed corn, molasses, calcium carbonate (limestone) 0.5 per cent, and iodized salt 0.5 per cent. No changes were made in feeding on the days of counting.

Leukocyte counts were made during the same general period of the day, beginning at approximately 9 to 9:45 a.m., with hourly determinations through 3:45 to 4 p.m. All total and differential examinations were made by one individual. Special consideration was given to the handling of the rabbits and all other procedures dealing with the collection and examination of blood samples to ensure the utmost accuracy of the results.

Blood was obtained from the lateral marginal vein of the left ear, punctured by a Hagedorn needle. Freely flowing drops of blood were considered essential. The same certified pipette was used for an animal throughout a day of counting. The diluting fluid was 1 per cent acetic acid, freshly prepared and filtered. Pipettes were shaken by hand for 3 minutes and discharged into duplicate counting chambers after the first three or four drops had been discarded. Certified Zeiss chambers with the Neubauer ruling and polished glass shoulders were used, and coverglasses were carefully adjusted so that proper depth of the counting chambers was obtained. Approximately 10 minutes was allowed for settling of the leukocytes.

The cells in the four large corner squares of both sides of two chambers were counted, so that 800 cells were actually enumerated when the total number of leukocytes was 10,000 per cu. mm. Thus in each case sixteen unit areas of 1 sq. mm. each were examined.

At least three blood smears were prepared for each hourly count. Smears were made on slides which had been freshly cleaned with acid alcohol and polished with a clean dry towel. Films were stained with Wright's stain and studied with the oil immersion objective, at a magnification of $\times$ 970. In each case, 300 leukocytes were counted, essentially the same areas being examined on each slide.

Leukocytes were classified into five general groups: polymorphonuclear neutrophils (amphophils), lymphocytes, large

mononuclear cells (monocytes), eosinophils, and basophils. In the present study, neutrophils (amphophils) were not subdivided into classes according to the appearance of the nuclear mass. The number of unclassified cells was less than 0.5 per cent in each case. Confusion was only occasionally encountered between lymphocytes and large mononuclear cells.

Total leukocyte counts were recorded to the nearest hundred. Differential percentages were adjusted to the nearest integer. Averages of the hourly total and absolute leukocyte counts and differential percentages of all rabbits were calculated by averaging counts made at seven consecutive hourly periods (from 9:45 to 10:05 a.m. through 3:45 to 4:05 p.m.). Averages of all counts of the day for each rabbit were also obtained. Morning and afternoon averages for individual rabbits were obtained by averaging all determinations during each respective period. In Table VI, differences between morning and afternoon averages are expressed as percentages of the morning average in each case.

TABLE I

Differential Leukocyte Examinations of 14 Normal Rabbits

Rabbit letter	Times of feeding and counts	Total leukocytes per cu. mm.	Differential percentages (300 leukocytes)				
			Neutrophils (amphophils)	Lymphocytes	Large mononuclear cells	Eosinophils	Basophils
	9:15 a.m.*						
	9:25 a.m.	7000	22	71	2	0	6
	10:25 a.m.	8800	21	71	1	1	6
	11:25 a.m.	7700	34	60	2	1	3
A	12:25 p.m.	6600	32	60	3	1	5
	1:25 p.m.	11900	34	61	1	0	4
	2:25 p.m.	12200	33	65	1	1	1
	3:25 p.m.	14900	38	57	1	1	3
	9:15 a.m.*						
	10:00 a.m.	7000	13	81	2	0	3
	11:00 a.m.	5900	14	80	2	1	3
B	12:00 noon	5100	13	82	2	0	3
	1:00 p.m.	6500	25	65	2	3	5
	2:00 p.m.	7000	20	74	1	0	4
	3:00 p.m.	7900	49	45	2	1	4
	11:00 a.m.*						
	9:45 a.m.	6000	10	86	2	0	2
	10:45 a.m.	5600	16	81	1	0	2
	11:45 a.m.	5800	23	73	0	0	3
B	12:45 p.m.	9500	46	51	1	0	2
	1:45 p.m.	7100	33	62	1	1	3
	2:45 p.m.	6700	56	39	0	0	4
	3:45 p.m.	6700	52	43	1	0	4

* Indicates time of feeding.

TABLE I—(Continued)

Rabbit letter	Times of feeding and counts	Total leukocytes per cu. mm.	Differential percentages (300 leukocytes)				
			Neutrophils (amphophils)	Lymphocytes	Large mononuclear cells	Eosinophils	Basophils
	9:30 a.m.*						
	10:00 a.m.	11800	34	56	2	1	8
	11:00 a.m.	8600	27	64	1	2	5
C	12:00 noon	7700	36	54	2	1	6
	1:00 p.m.	8600	45	46	1	1	8
	2:00 p.m.	11500	41	43	1	0	15
	3:00 p.m.	7200	39	47	1	1	12
	9:40 a.m.*						
	9:00 a.m.	7300	11	86	1	0	2
	10:00 a.m.	6900	23	70	1	1	5
D	11:00 a.m.	7500	22	71	2	1	4
	12:00 noon	10400	33	64	1	0	2
	1:00 p.m.	7700	39	55	2	0	4
	2:00 p.m.	8400	38	56	2	1	3
	10:00 a.m.*						
	9:55 a.m.	5500	41	55	1	1	2
	10:55 a.m.	5600	35	60	1	1	3
	11:55 a.m.	6300	31	62	3	1	4
E	12:55 p.m.	11100	35	62	0	2	2
	1:55 p.m.	4600	34	60	1	2	3
	2:55 p.m.	6300	49	46	1	0	4
	3:55 p.m.	8200	55	40	0	0	5
	9:30 a.m.*						
	10:05 a.m.	10600	23	72	1	1	2
	11:05 a.m.	7900	23	75	1	0	1
F	12:05 p.m.	9700	21	72	1	2	4
	1:05 p.m.	8900	17	80	1	0	2
	2:05 p.m.	10200	24	72	1	1	2
	3:05 p.m.	11000	29	64	1	0	6
	4:05 p.m.	10800	37	56	1	1	4
	10:30 a.m.*						
	9:45 a.m.	11300	24	70	4	0	2
	10:45 a.m.	10200	36	58	3	0	2
G	11:45 a.m.	9600	33	56	5	0	6
	12:45 p.m.	8900	31	60	3	0	6
	1:45 p.m.	10600	37	55	3	1	5
	2:45 p.m.	8300	32	63	3	0	3
	3:45 p.m.	8900	29	63	2	0	6
	10:00 a.m.*						
	9:45 a.m.	8200	41	53	2	0	4
	10:45 a.m.	9000	39	56	1	1	4
H	11:45 a.m.	9800	39	55	1	0	5
	12:45 p.m.	8300	39	53	1	1	6
	1:45 p.m.	7900	45	49	0	1	5
	2:45 p.m.	9200	50	42	0	1	7
	3:45 p.m.	8300	47	50	0	0	3

* Indicates time of feeding.

TABLE I—(Continued)

Rabbit letter	Times of feeding and counts	Total leukocytes per cu. mm.	Differential percentages (300 leukocytes)				
			Neutrophils (amphophils)	Lymphocytes	Large mononuclear cells	Eosinophils	Basophils
	10:00 a.m.*						
	9:45 a.m.	12200	11	87	1	0	1
	10:45 a.m.	10600	11	84	2	0	3
I	11:45 a.m.	10100	14	82	2	0	3
	12:45 p.m.	11600	12	76	3	1	8
	1:45 p.m.	9000	18	72	2	1	7
	2:45 p.m.	10500	18	76	1	2	4
	3:45 p.m.	10500	15	80	1	0	3
	9:30 a.m.*						
	10:45 a.m.	9300	21	69	2	0	7
	11:45 a.m.	9800	27	65	2	2	5
J	12:45 p.m.	11700	28	63	2	1	5
	1:45 p.m.	11600	33	59	1	2	6
	2:45 p.m.	10000	34	57	2	1	7
	3:45 p.m.	9300	37	54	3	0	6
	11:00 a.m.*						
	9:45 a.m.	12500	21	75	2	1	1
	10:45 a.m.	12100	21	71	3	0	5
K	11:45 a.m.	15700	25	68	1	0	5
	12:45 p.m.	14200	34	60	2	0	4
	1:45 p.m.	10200	43	53	1	1	2
	2:45 p.m.	12600	40	53	1	1	5
	3:45 p.m.	10900	29	65	1	0	5
	11:00 a.m.*						
	9:55 a.m.	15800	38	56	1	1	4
	10:55 a.m.	16200	39	57	3	0	1
L	11:55 a.m.	14300	45	45	3	1	6
	12:55 p.m.	16200	48	47	2	1	3
	1:55 p.m.	13700	40	57	1	0	1
	2:55 p.m.	14300	44	52	1	0	3
	3:55 p.m.	14600	44	53	1	0	2
	10:30 a.m.*						
	9:45 a.m.	9100	5	90	0	0	4
	10:45 a.m.	15000	14	83	1	0	2
M	11:45 a.m.	13800	15	81	0	0	4
	12:45 p.m.	9900	30	66	1	0	3
	1:45 p.m.	9800	36	58	1	1	4
	2:45 p.m.	10500	25	70	1	1	4
	3:45 p.m.	10900	21	76	0	0	2
	10:30 a.m.*						
	9:55 a.m.	5200	13	70	3	3	11
	10:55 a.m.	5600	27	65	3	0	5
N	11:55 a.m.	6600	38	53	1	1	7
	12:55 p.m.	5700	44	47	1	3	4
	1:55 p.m.	6700	51	43	0	1	4
	2:55 p.m.	6400	38	50	1	2	9
	3:55 p.m.	8700	48	46	1	1	4

* Indicates time of feeding.

Results

Results are presented in tabular form. Table I shows for each experiment the times of counts, the times that food was placed in the rabbit cages, total numbers of leukocytes per cu. mm. and differential percentages of neutrophils (amphophils), lymphocytes, large mononuclear cells, eosinophils, and basophils. As may be seen, there is considerable daily variation in different experiments and the time of placing food in the cages seems to bear no direct relationship to these variations. Variations in averages of all counts of the day of each rabbit, presented in Table II, likewise are of considerable magnitude. Repeated leukocyte examinations of 3 animals at approximately the same time on different days, prior or subsequent to the day of hourly counting, tend to show that, in general, each rabbit seems to have its own characteristic leukocyte picture and variations appear to be decreased under such conditions (Table III). Repeated leukocyte examinations of the other rabbits showed similar results.

Table II

Averages of Total Leukocyte Counts, Differential Percentages and Absolute Numbers of Leukocytes for All Counts of the Day of 14 Normal Rabbits

Rabbit letter	Average total leukocytes per cu. mm.	Average differential percentages and absolute numbers of leukocytes per cu. mm.			
		Neutrophils (amphophils)		Lymphocytes	
		Per cent	Absolute number	Per cent	Absolute number
A	9871	30	3122	63	6211
B	6566	22	1549	71	4579
B	6771	33	2397	62	4095
C	9233	37	3416	52	4759
D	8033	27	2277	67	5338
E	6800	40	2744	55	3730
F	9871	25	2491	70	6870
G	9685	31	3067	61	5886
H	8671	43	3712	51	4437
I	10642	14	1485	79	8496
J	10283	30	3090	61	6287
K	12600	30	3786	63	8047
L	15014	42	6389	52	7873
M	11285	21	2291	75	8524
N	6414	37	2461	53	3362
Average	9449	31	2952	62	5900

Averages of the hourly total and absolute leukocyte counts and differential percentages of all 14 rabbits (Table IV) fail to reflect

TABLE III

Repeated Leukocyte Examinations of 3 Normal Rabbits at Approximately the Same Time of Day on Different Days

Rabbit letter	Time	Total leukocytes per cu. mm.	Neutrophils (amphophils)		Lymphocytes	
			Per cent	Absolute number	Per cent	Absolute number
B	10:00 a.m.	9500	11	1045	83	7885
	10:30 a.m.	7200	11	792	84	6048
	10:20 a.m.	7000	5	350	88	6160
	10:00 a.m.	7000	13	910	81	5670
	9:45 a.m.	6000	10	600	86	5160
	9:40 a.m.	6300	8	504	87	5481
I	9:45 a.m.	12200	11	1342	87	10614
	9:05 a.m.	11300	7	791	87	9831
	9:30 a.m.	14400	7	1008	86	12384
	9:42 a.m.	14700	8	1176	87	12789
	9:45 a.m.	12700	8	1016	89	11303
N	9:55 a.m.	5200	13	676	70	3640
	8:55 a.m.	7100	22	1562	66	4686
	8:55 a.m.	5700	9	513	81	4617
	9:25 a.m.	6000	13	780	76	4650
	9:55 a.m.	5800	22	1276	63	3654

TABLE IV

Averages of Hourly Leukocyte Counts of 14 Normal Rabbits

Time of count	Number of determinations averaged	Average total leukocytes per cu. mm.	Average differential percentages and absolute numbers of leukocytes per cu. mm.			
			Neutrophils (amphophils)		Lymphocytes	
			Per cent	Absolute number	Per cent	Absolute number
9:45 a.m. to 10:05 a.m.	12	9600	23	2282	71	6751
10:45 a.m. to 11:05 a.m.	14	9221	24	2303	69	6397
11:45 a.m. to 12:05 p.m.	14	9621	28	2727	65	6237
12:45 p.m. to 1:05 p.m.	14	9914	33	3358	59	5895
1:45 p.m. to 2:05 p.m.	14	9164	35	3242	58	5295
2:45 p.m. to 3:05 p.m.	13	9300	38	3511	54	5148
3:45 p.m. to 4:05 p.m.	11	9800	38	3594	56	5681

the extent of the variations evident from the previous tables dealing with individual animals. When only average determinations are considered, in general the total leukocyte values are fairly constant, the absolute number of lymphocytes slightly decreases and the absolute number of neutrophils (amphophils) slightly increases during the day. Consequently, the differential percentages of lymphocytes and neutrophils (amphophils) slightly decrease and slightly increase, respectively. Percentages and absolute numbers of large mononuclear cells, eosinophils and basophils fluctuate but cannot be considered to exhibit significant trends because these cells comprise only a small fraction of the circulating leukocyte population.

TABLE V

*Increase or Decrease of Afternoon Average Leukocyte Counts, with Reference to Morning Average Leukocyte Counts, in 14 Normal Rabbits**

Rabbit letter	Total leukocytes	Neutrophils (amphophils)	Lymphocytes
A	+3567	+1959	+1664
B	+ 175	+1022	− 910
B	+1700	+2544	− 958
C	−1450	+ 374	−1945
D	+1600	+1853	− 280
E	+1750	+1205	+ 525
F	+ 870	+ 510	+ 129
G	−1191	− 205	− 900
H	− 575	+ 258	− 854
I	− 566	+ 312	−1347
J	+1100	+1187	− 159
K	−1458	+1324	−2625
L	− 733	+ 241	− 523
M	−2358	+1311	−3643
N	+1075	+1568	− 403

* A positive sign indicates that the afternoon average was greater than the morning average by the total difference indicated. A negative sign indicates that the afternoon average was less than the morning average by the total difference indicated.

The increase or decrease of the average of the afternoon total leukocyte counts of individual rabbits, with reference to the average of the morning determinations in each case, is shown in Table V. It is evident that an apparent increase in the average of the afternoon total leukocyte counts was a fairly frequent but not constant finding. These increases or decreases are expressed as percentages of the respective morning averages in each case in Table VI. In this table the afternoon leukocyte fluctuations noted in studies of normal human leukocyte variations by Sabin and coworkers,[1] Shaw,[8] and Ponder, Saslow and Schweizer,[6] and ex-

pressed as percentage increases or decreases (with reference to respective morning averages) by Ponder and co-workers,[6] are compared with similarly expressed percentage increases or decreases in individual rabbits in the present study. Although comparison of human and rabbit experiments may be unjustifiable, it should be emphasized that expression of differences between two variables in terms of percentage of one of these variables may introduce confusion in interpretation of findings. Since biometric analyses seem unjustified in view of the small number of variants in the present study, actual numerical increases or decreases of the individual afternoon averages are listed in Table V.

Further examination of Table V shows that, in the 14 rabbits studied, an apparent afternoon increase in the number of neutrophils (amphophils) appeared to be more frequent than an afternoon rise in the total leukocyte count. An apparent afternoon decrease in the number of lymphocytes seemed to be a fairly frequent, although inconstant, finding. Variations in numbers of blood lymphocytes may, to a large extent, reflect similar changes in lymph flow.[4]

From a practical standpoint, these results seem to indicate that when the hourly leukocyte picture of the rabbit is studied under different experimental conditions, it would seem advisable to de-

TABLE VI

Increase or Decrease of Afternoon Average Total Leukocyte Counts, Expressed as Percentages of Respective Morning Average Leukocyte Counts, in Normal Human Subjects and in 14 Normal Rabbits

Subject	Percentage relationship as found by different authors			
	Normal human subjects			14 normal rabbits
	Ponder *et al.*[6]	Shaw[8]	Sabin *et al.*[1]	Reifenstein *et al.*
1	3	26	17	45
2	1	15	15	−13
3	12	−13	14	22
4	4	28	9	30
5	10	30	6	9
6	−24	13	24	−11
7	57	35	..	− 6
8	13	20	..	− 5
9	..	3	..	11
10	..	15	..	−11
11	..	..	..	− 5
12	..	..	..	−18
13	..	..	..	18
14a*	..	..	..	3
14b*	..	..	..	29

* 14a and 14b represent the same rabbit (B) counted on two occasions.

termine the general normal leukocyte trends of each animal and then maintain the same period of the day for counting at hourly intervals under experimental conditions, as well as to establish similar control of other possible sources of error in the determinations.

Summary

Hourly total and differential leukocyte studies of 14 normal rabbits are presented.

Individual examinations show considerable variation; average counts exhibit tendencies for the total numbers of leukocytes to vary little, and for the percentages and absolute numbers of lymphocytes and neutrophils (amphophils) to decrease and increase slightly, respectively, during the day.

An apparent afternoon increase in the number of neutrophils (amphophils) is a more frequent finding than an apparent afternoon increase in the total leukocyte count.

In studying the hourly leukocyte picture of rabbits under different experimental conditions, it would seem advisable to determine general normal leukocyte trends for each animal and then establish the same periods of the day for making counts under experimental conditions.

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STUDIES ON LEUKOCYTOSIS *

II. Neutrophilic Leukocytosis Following Intravenous Injection of Supernatant Fluid from a Sterile Exudate (Rabbit)

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It is generally postulated that the polymorphonuclear neutrophilic leukocytosis associated with acute inflammation is due to the presence in the inflamed area of some substance which enters the blood stream and effects a transient increase in the number of leukocytes (polymorphonuclear neutrophils) in the circulating blood. This increase apparently occurs rather rapidly during the course of several hours. Such a neutrophilic increase may be accompanied by a normal total leukocyte count (*relative neutrophilic leukocytosis*), or may be associated with a variable increase in the total leukocyte count hereafter referred to as an *absolute neutrophilic leukocytosis*.

If such a substance exists, the intravenous injection into one animal of a relatively small amount of sterile exudate, of less than 24 hours' duration in an homologous animal, should be followed by a transient increase in the number of polymorphonuclear neutrophilic leukocytes in the circulating blood of the injected animal. Such a neutrophilic rise should occur a relatively short time after injection. Perhaps in some instances an absolute neutrophilic leukocytosis might occur.

In the present study of experimental leukocytosis, sterile procedures were employed throughout all experiments. Sterile 0.9 per cent saline solution was injected intraperitoneally into rabbits to produce peritoneal exudates. In each experiment this exudate was withdrawn 5¾ to 12 hours later, centrifugalized and its

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supernatant fluid fraction retained. In a series of 13 experiments with 9 rabbits, each rabbit received the same amount (5 cc.) of such supernatant fluid by intravenous injection. Total and differential leukocyte studies of these rabbits were made at hourly intervals after the intravenous injection. These observations were compared with similar leukocyte determinations following intravenous injections of similar amounts of sterile saline solutions, of the same chloride concentrations as the supernatant fluid fractions, using the same experimental animals on five occasions.

Procedure

The method used for the production of exudates was similar to those of de Haan;[1] Mudd, Lucké, McCutcheon and Strumia;[2] Ponder and Macleod;[3] and Coman.[4] A saline solution was prepared by dissolving 9 gm. of sodium chloride, C. P. (Merck), in water doubly distilled from glass, to a total volume of 1 L. The solution was autoclaved at 15 pounds pressure for 30 minutes. The P_H of this solution before and after sterilization was approximately 6.2, as determined colorimetrically and by the quinhydrone electrode. Approximately 300 cc. of this sterile solution was injected at room temperature, without anesthesia and by gravity flow, into the peritoneal cavity of a rabbit. After 5¾ to 12 hours, the fluid exudate was withdrawn under sterile conditions without anesthesia, using a large bore needle, rubber tubing and a glass suction bottle. The periods of exudation occurred at various times of the day and night. Repeated exudates were produced in some animals, but the shortest period of time between intraperitoneal injections in the same rabbit was 10 days. No essential differences were noted between the first and subsequent exudates but occasionally, after repeated injections, fibrous adhesions in the peritoneal cavity seemed to hinder removal.

Repeated bacteriological examinations of sterilized saline, whole exudates and supernatant fluids, using direct smears, blood plates, infusion agar and corn syrup agar, yielded negative results.

The amount of exudate obtained varied up to 154 cc., apparently depending on the duration of exudation and the individual rabbit. Several times when no fluid was obtained, none could be found upon immediate postmortem examination of the animal. In these cases there were no indications that the fluid had been

injected into the urinary bladder or gastro-intestinal tract. Of 75 instances in which an attempt was made to withdraw fluid, no appreciable exudate could be obtained in 15.

The exudate was colorless, opalescent, cloudy or grossly purulent and sometimes contained small flecks of fibrinous material. Occasionally the fluid clotted immediately or shortly after removal. The specific gravity ranged from 1.012 to 1.022. The total leukocytes per cu. mm. varied from several hundred to 33,000, of which approximately 80 to 95 per cent were polymorphonuclear neutrophils (amphophils) and the remainder were lymphocytes and monocytes.

After samples were taken for leukocyte counts and bacteriological examinations, the sterile exudate was immediately centrifugalized for 15 minutes at a speed of 3125 r. p. m. Fibrin clots adherent to the centrifuge tube were discarded. The supernatant fluid was carefully decanted or pipetted off. Five cc. of this fluid was immediately injected intravenously into the rabbit to be studied, using the lateral marginal vein of the previously shaved right ear. In performing the injection, care was taken to avoid infiltration of fluid into the ear, thrombosis or excessive trauma, and the vein was examined for patency and freedom of venous flow following the injection.

Nine rabbits were used in the present experiments. Each rabbit received one intravenous injection of 5 cc. of supernatant fluid and 4 of the 9 (animals B, F, I and K) received a second such injection after an interval of 58 to 106 days.

Using Whitehorn's [5] method, determinations were made of the chloride concentrations of the supernatant fractions of the centrifugalized exudates. They varied between 0.90 and 0.78 gm. of sodium chloride per 100 cc. of supernatant fluid. As a control procedure, sterile sodium chloride solutions, containing 0.90 to 0.78 gm. of sodium chloride per cent, were prepared in the described manner and injected intravenously into 5 of the 9 rabbits in an equivalent amount (5 cc.).

The various procedures involved in leukocyte determinations, such as feeding, housing, selection of animals, obtaining of blood samples and actual counting, have been described previously.[6] Special consideration was given to the exact duplication of these methods and to all other procedures to ensure the utmost accuracy

TABLE I

Results of All Experiments on Leukocytosis (9 Rabbits)

Rabbit letter	Experiment number	Experimental procedure	Hourly total leukocyte counts per cu. mm.							Hourly neutrophil (amphophil) percentages							Hourly lymphocyte percentages						
			1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
B	39.10	No injection	7000	5900	5100	6500	7000	7900	....	13	14	13	25	20	49	..	81	80	82	65	74	45	..
B	39.39	No injection	6000	5600	5800	9500	7100	6700	6700	10	16	23	46	33	56	52	86	81	73	51	62	39	43
B	39.48	Super. fluid	7100	6800	12600	10300	8300	8100	8800	18	38	80	71	71	65	65	74	56	17	25	24	30	26
B	40.22	Super. fluid	6300	5900	9300	8400	7500	8500	6900	8	30	78	67	75	65	71	87	65	19	26	23	30	26
B	39.2	0.90% NaCl	6000	5900	7200	6400	7500	6200	6900	15	13	34	48	46	40	38	77	81	56	35	43	46	53
F	39.33	No injection	10600	7900	9700	8900	10200	11000	10800	23	23	21	17	24	29	37	72	75	72	80	72	64	56
F	39.47	Super. fluid	7500	5100	12900	11700	9700	9700	10600	20	14	44	63	50	51	47	71	79	44	29	42	39	46
F	40.26	Super. fluid	6700	8200	10500	10000	9300	9300	8200	17	20	54	40	44	35	44	74	69	40	51	49	60	49
F	40.30	0.78% NaCl	8000	8800	10000	7400	10100	8400	7000	21	28	23	30	30	26	32	75	70	76	68	67	72	66
H	39.40	No injection	8200	9000	9800	8300	7900	9200	8300	41	39	39	39	45	50	47	53	56	55	53	49	42	50
H	39.49	Super. fluid	8200	14500	29900	32000	26100	25300	24800	9	15	38	42	42	38	31	88	84	60	54	53	59	65
I	39.43	No injection	12200	10600	10100	11600	9000	10500	10500	11	11	14	12	18	18	15	87	84	82	76	72	76	80
I	40.1	Super. fluid	14400	14400	11200	11900	13500	10600	13200	7	15	47	34	28	32	19	86	73	50	61	66	57	77
I	40.24	Super. fluid	14700	13100	15600	15700	15400	14500	18300	8	10	28	26	30	15	21	87	87	68	72	65	81	76
I	40.29	0.78% NaCl	12700	10300	10700	14300	16500	14900	15700	8	6	17	19	16	11	18	89	91	79	79	83	88	79
J	39.44	No injection	....	9300	9800	11700	11600	10000	9300	..	21	27	28	33	34	37	..	69	65	63	59	57	54
J	40.2	Super. fluid	12700	13300	14000	12500	12700	12900	12100	10	24	55	49	49	51	43	83	65	39	46	45	43	46
K	40.4	No injection	12500	12100	15700	14200	10200	12600	10900	21	21	25	34	43	40	29	75	71	68	60	53	53	65
K	40.8	Super. fluid	11200	8600	10900	11200	12100	11700	13400	13	32	55	54	52	45	44	76	64	43	45	41	49	45
K	40.23	Super. fluid	11500	9200	6400	10200	10700	10100	15400	20	37	59	68	51	61	45	77	59	36	30	44	34	49
L	40.5	No injection	15800	16200	14300	16200	13700	14300	14600	38	39	45	48	40	44	44	56	57	45	47	57	52	53
L	40.9	Super. fluid	12400	8200	12600	16800	12800	16500	12600	26	33	57	67	58	69	72	68	64	41	29	38	25	24
M	40.6	No injection	9100	15000	13800	9900	9800	10500	10900	5	14	15	30	36	25	21	90	83	81	66	58	70	76
M	40.10	Super. fluid	12900	11300	13000	14200	13500	13300	11800	13	37	39	45	44	43	36	83	59	57	52	50	54	60
M	40.31	0.85% NaCl	12200	13100	12200	14100	13000	13200	14200	10	7	9	20	19	16	18	86	90	89	76	79	83	80
N	40.7	No injection	5200	5600	6600	5700	6700	6400	8700	13	27	38	44	51	38	48	70	65	53	47	43	50	46
N	40.11	Super. fluid	7100	9900	10900	9700	8800	9900	8000	22	46	53	47	47	42	37	66	43	35	41	44	47	52
N	40.32	0.85% NaCl	5800	5600	6400	7900	8500	11200	11900	22	19	17	30	39	35	42	63	76	75	67	55	56	53

of the results. Hourly leukocyte variations of each animal were determined previous to any experimental procedures involving it and, as far as possible, the same times of counting were maintained when the leukocytes were studied after the animal had been injected. In all experiments, leukocyte counts were made at approximately hourly intervals through the sixth hour following injection. In 11 of the 13 experiments, determinations were made also 24 to 31 hours after injection.

Results

All results for each of the 9 rabbits are presented in Table I. Hourly total leukocyte counts and neutrophil (amphophil) and lymphocyte percentages are shown for each experimental procedure performed on each rabbit. Thus, the leukocyte picture of rabbit B was studied twice without injection (experiments Nos. 39.10 and 39.39), twice following an intravenous injection of 5 cc. of supernatant fluid from a sterile exudate (experiments Nos. 39.48 and 40.22), and once following an intravenous injection of 5 cc. of 0.9 per cent sterile sodium chloride solution (experiment No. 39.2).

With rabbit B, both intravenous injections of supernatant fluid were followed in 2 hours by absolute neutrophilic leukocytoses which were maintained for at least 6 hours after injection. The percentage of neutrophils increased in 3 hours from 18 to 80 in experiment No. 39.48, and from 8 to 78 in experiment No. 40.22. During these 3 hours the absolute number of neutrophils increased from 1,278 to 10,080 in the former experiment, and from 504 to 7,254 in the latter. The absolute number of neutrophils found in the circulating blood of this animal without injection, at the time of day corresponding to the time of the neutrophil peaks, was 663 in one experiment and 1,334 in the other. The increase in the total leukocyte count, although not marked in either instance, appeared to be definitely greater than the normal hourly total leukocyte fluctuations of this animal.

Although it may seem that the lymphocytes of rabbit B decreased almost as much as the neutrophils increased following injection in experiment No. 39.48, this lymphocytic decrease is no greater than the lymphocytic decrease which normally occurred in this animal during the same period of the day without injection.

N	40.32	0.85% NaCl	5800	5600	6400	7900	8500	11200	11900	22	19	17	30	39	35	42	63	76	75	67	55	56	53

This fact is more evident in Chart 1, in which the leukocyte curves of rabbit B without injection (experiment No. 39.10), following supernatant fluid injection (experiment No. 39.48) and following sterile saline injection (experiment No. 39.2) are compared.

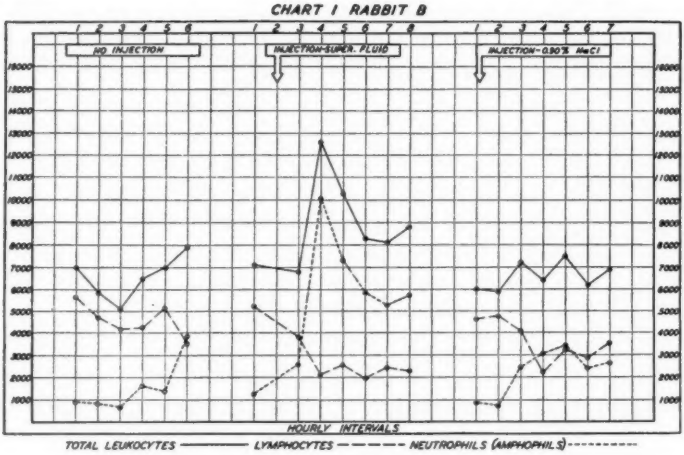
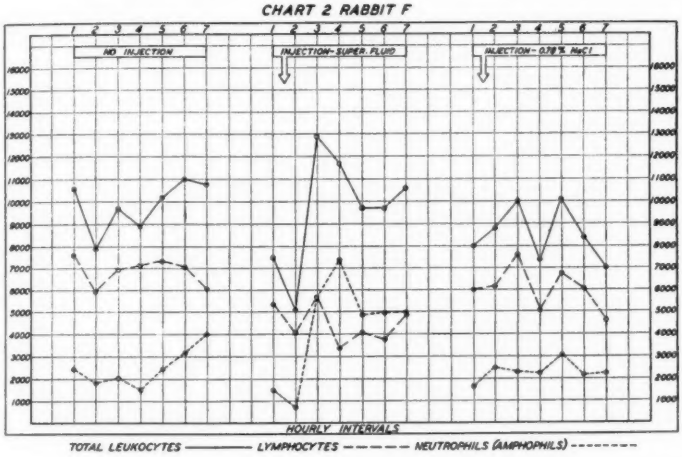


Chart 1 clearly indicates the sharply peaked neutrophilic leukocytosis which occurred 2 hours following the injection of supernatant fluid. The rise in total leukocyte count was apparently due entirely to the rapid increase in neutrophils, and the subsequent variations in the total leukocyte count reflected similar neutrophil variations.

Table I shows similar neutrophilic leukocytoses following intravenous injections of supernatant fluid into rabbit F. With this animal the neutrophil peak occurred 2½ hours after injection in experiment No. 39.47 and 2 hours after injection in experiment No. 40.26. The increases in neutrophil percentages over respective periods of 3 and 4 hours were from 20 to 63 and from 17 to 54. The increase in absolute number of neutrophils was from 1,500 to 7,371 in experiment No. 39.47 and from 1,139 to 5,670 in experiment No. 40.26. The normal absolute neutrophil numbers, at times corresponding to the times of each neutrophil peak, were 1,513 and 2,037 respectively. While the neutrophilic leuko-

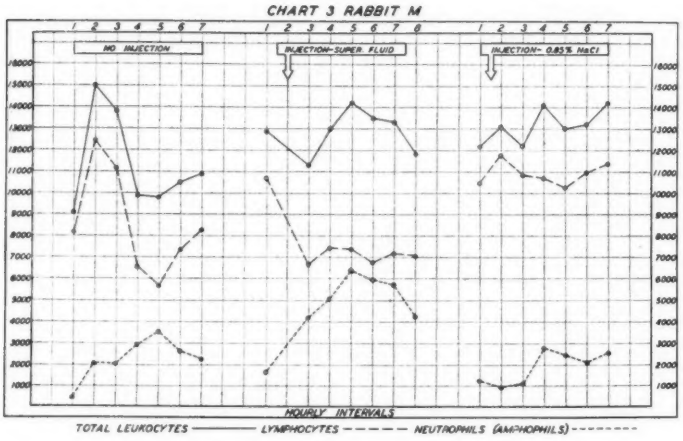
cytosis was sharply peaked in both these experiments, an absolute neutrophilic leukocytosis occurred only in experiment No. 39.47. Chart 2 compares the leukocyte curves of rabbit F without injection (experiment No. 39.33), following supernatant fluid injection (experiment No. 39.47) and following sterile saline in-



jection (experiment No. 40.30). It is evident that the hourly absolute number of lymphocytes tended to remain fairly constant in all experiments performed on this animal.

After comparison of the leukocyte curves for the three experiments performed on rabbit M (Table I and Chart 3), it is evident that this animal always had a total leukocyte level of 10,000 to 15,000 per cu. mm. through the periods of counting. This has been confirmed by repeated leukocyte examinations of this animal without injection at approximately the same time of day on different days. Chart 3 shows that the sudden variations in the total leukocyte count, which occurred when the animal was examined hourly without injection (experiment No. 40.6), apparently were due to similar variations in the absolute number of lymphocytes. The absolute number of neutrophils increased slightly during the period of counting without injection, consistent with the hourly neutrophil variations of normal rabbits. Three hours fol-

lowing the intravenous injection of supernatant fluid into rabbit M (experiment No. 40.10), a peaked neutrophilic leukocytosis occurred. During a 4 hour period the percentage of neutrophils increased from 13 to 45 and the absolute number of neutrophils rose from 1,677 to 6,390. This neutrophil peak was not as sharp as those of rabbits B and F in Charts 1 and 2 respectively, but it



occurred at approximately the same time after injection and was of sufficient magnitude to exceed considerably the normal absolute number of neutrophils at a corresponding hour of the day (2,970). No absolute neutrophilic leukocytosis occurred in this animal after the injection of supernatant fluid.

The results obtained with the remaining rabbits are tabulated in Table I. An absolute neutrophilic leukocytosis occurred in 5 of the 13 experiments (Nos. 39.47, 39.48, 39.49, 40.11 and 40.22), and was suggestive in another experiment (No. 40.26). However, the absolute numbers of neutrophils increased in all experiments following the injection of supernatant fluid. The average increase in absolute numbers of neutrophils from before supernatant fluid injection to the time of each neutrophil peak was 6,086; the corresponding average increase in neutrophil percentage was 42. The average increase in absolute numbers of neutrophils in the same animals without injection, during this same period of the day, was 1,389 and the corresponding average increase in neutrophil

percentage was 10. In every experiment, the absolute numbers of lymphocytes, large mononuclear cells, eosinophils and basophils showed insignificant variations which were no greater than the hourly leukocyte variations of these animals, examined without injection.

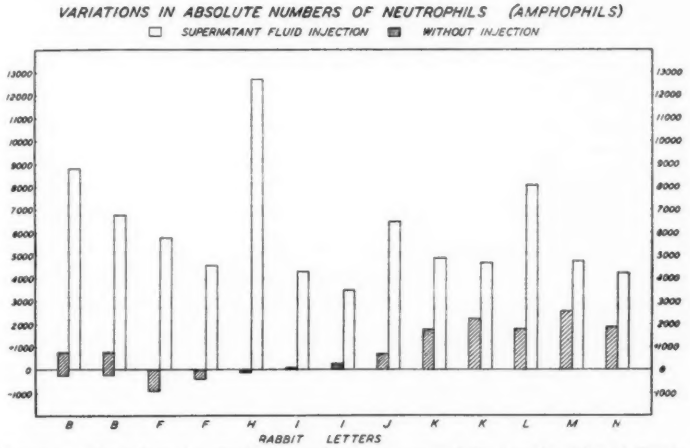


CHART 4. Variations in absolute numbers of neutrophils (amphophils) following supernatant fluid injection and without injection in the same animals. The blank column labelled "supernatant fluid injection" indicates the increase in absolute numbers of neutrophils (amphophils) which occurred from the time previous to the injection of supernatant fluid to the time of the neutrophil peak after the injection. The diagonally-crossed column labelled "without injection" indicates the increase or decrease in the absolute numbers of neutrophils (amphophils) which occurred in the same animals during a corresponding period of the day.

Chart 4 compares the variations in absolute numbers of neutrophils, from a time previous to injection to the time of the neutrophil peak following supernatant fluid injection, of each of the 9 rabbits, with the neutrophil variations of the same animals without injection during a corresponding period of the day. Except for 3 animals(K, M and N), the neutrophil increases following the injection of supernatant fluid considerably exceeded the normal neutrophil fluctuations during corresponding periods of the day in each case. In 3 animals (B, F and H), the absolute numbers of neutrophils decreased without injection during a period of the day comparable to the interval from before injection to the neutrophil peak.

Rabbits K, M and N showed the greatest fluctuation in absolute numbers of neutrophils of any of the 9 rabbits studied without injection. However, the neutrophil increases of rabbits K, M and N following supernatant fluid injection reached levels comparable to the neutrophil increases following such injection in the other 6 animals. Furthermore, Chart 3 shows that a neutrophil peak occurred 3 hours after rabbit M had received supernatant fluid. A similar neutrophil peak occurred 2 hours after supernatant fluid injection in rabbit N, and 3 hours after injection in rabbit K. At the times of these peaks the absolute numbers of neutrophils present in the circulating blood of these 3 animals were considerably in excess of the absolute number of neutrophils present in the blood of these animals without injection at times comparable to those of the neutrophil peaks.

Table II shows for all experiments the absolute numbers and percentages of neutrophils before injection, at the neutrophil peak, and without injection at corresponding times. The number of hours after injection that each neutrophil peak occurred is also indicated. It is evident that the neutrophil peak occurred most frequently (in 12 of the 13 experiments) at the second or third hour after injection. A secondary neutrophil peak occurred in 2 experiments (Nos. 40.9 and 40.24).

The duration of the exudate from which each supernatant fluid fraction was obtained is also indicated in Table II. In this series of 13 experiments, no differences were noted between the leukocyte counts of the 2 experiments with supernatant fractions of exudates of 5¾ hours' duration and the leukocyte counts of the 11 experiments with supernatant fractions of exudates of from 9½ to 12 hours' duration.

In 4 of the 13 experiments, normal total and absolute leukocyte levels were reached 6 hours after injection. In the remaining 7 experiments in which leukocyte examinations were made more than 6 hours after injection, normal total and absolute leukocyte counts were present 24 to 31 hours after injection.

Five of the 9 rabbits (B, F, I, M and N) received an intravenous injection of 5 cc. of sterile sodium chloride solutions having chloride concentrations the same as those of the supernatant fluid fractions of the centrifugalized exudates. Charts 1, 2 and 3 indicate that the leukocyte pictures following sterile saline injec-

TABLE II
Characteristics of Neutrophilic Leukocytoses in 13 Experiments

Rabbit letter	Experiment number	Duration of exudate	Absolute numbers of neutrophils (amphophils)				Neutrophil (amphophil) percentages				Neutrophil peak (hours after injection)	Absolute neutrophilic leukocytosis	Return to normal level (hours after injection)
			Before injection	Corresponding hour without injection	At neutrophil peak	Corresponding hour without injection	Before injection	Corresponding hour without injection	At neutrophil peak	Corresponding hour without injection			
		hours											
B	39.48	10¼	1278	910	10080	663	18	13	80	13	2	+	31
B	40.22	5¾	504	910	7254	663	8	13	78	13	2	+	Undetermined
F	39.47	9½	1500	2438	7371	1513	20	23	63	17	2½	+	26
F	40.26	12	1139	2438	5670	2037	17	23	54	21	2	?	6
H	39.49	10¼	738	3362	13440	3237	9	41	42	39	3	+	†
I	40.1	10½	1008	1342	5264	1414	7	11	47	14	2	−	6
I	40.24	12	1176	1342	4620	1620	8	11	30	18	2, 4	−	6
J	40.2	10½	1270	1953*	7700	2646	10	21	55	27	2	−	25
K	40.8	10¼	1456	2625	6292	4386	13	21	52	43	4	−	30
K	40.23	5¾	2300	2625	6936	4828	20	21	68	34	3	−	Undetermined
L	40.9	10¼	3224	6004	11256	7776	26	38	67	48	3, 5	−	30
M	40.10	10¼	1677	455	6390	2970	13	14	45	30	3	−	24
N	40.11	10¼	1562	676	5777	2508	22	13	53	38	2	+	6

* Approximate.
† Did not approach normal level 145 hours after injection; autopsy revealed otitis media.

tions of rabbits B, F and M respectively showed variations consistent with the fluctuations present in each of these animals without injection. Similar leukocyte fluctuations followed the sterile saline injection of 2 additional animals. In each of these 5 rabbits, the leukocyte variations following a control injection of sterile sodium chloride solution were no greater than the variations which occurred in the same animals studied without injection during a corresponding period of the day.

It is evident from Table II and Chart 4 that the results with rabbit H differed somewhat from those of the other 8 animals. In the first place, this animal, when examined without injection (experiment No. 39.40), had a relatively high percentage and absolute number of neutrophils. Following the intravenous injection of supernatant fluid from a sterile exudate (experiment No. 39.49), a peaked absolute neutrophilic leukocytosis occurred, the height of the neutrophilic increase being reached 3 hours after the injection. Leukocyte examinations made 4, 5, 6, 12, 31, 53, 96, 120 and 145 hours after the injection showed that normal leukocyte levels had not been reached at any time. Repeated examinations of this rabbit failed to reveal any abnormalities. The rectal temperature was consistently normal. The animal was killed 145½ hours after the supernatant fluid injection. An immediate autopsy showed purulent right otitis media. We feel that this infection was a possible cause of the atypical leukocyte counts obtained in this experiment.

Discussion

Various agents are known to produce polymorphonuclear neutrophilic leukocytosis. Among these may be mentioned bacteria and their products,[7] certain chemicals and drugs (such as histamine and epinephrine), nucleoproteins and some of their components, and various protein materials.[8] The mechanisms of action of these various leukocytosis-producing substances is a matter of some controversy and will not be considered in this report.

A number of substances have been postulated to be responsible for the polymorphonuclear neutrophilic leukocytosis of acute inflammation. Acute inflammation may be accompanied by marked polymorphonuclear leukocytosis with or without bacterial infection being present. As bacterial products *per se* are known to

cause leukocytosis, it is possible that the leukocytosis of acute inflammation often may be the result of action of more than one substance.

Many observations have shown that polymorphonuclear neutrophilic leukocytosis may occur after the injection of nucleic acid or some of its derivatives.[9-12] Intravenous injections into rabbits of approximately 1 gm. of nucleic acid or its salt, sodium nucleinate, frequently are followed by a preliminary period of leukopenia lasting approximately 5 or 6 to 24 hours. This leukopenic phase may be followed by an absolute polymorphonuclear (amphophil) leukocytosis which reaches its peak 7 or 8 to 24 hours after injection, with a return to normal leukocyte levels 3 or 4 days following the injection. A more rapidly developing absolute polymorphonuclear (amphophil) leukocytosis, with a peak several hours after injection and a return to normal leukocyte levels 8 to 48 or more hours after injection, frequently follows the intravenous injection into rabbits of approximately 1 gm. of nucleic acid derivatives, such as adenine or guanine nucleotides.[12,13] Doan, Zerfas, Warren and Ames [12] reported a similar prompt absolute polymorphonuclear (amphophil) leukocytosis following intravenous injection of approximately 1 gm. of sodium nucleinate into splenectomized rabbits. The initial leukopenic phase was absent in these accelerated leukocytoses.

The relatively prompt leukocytic response which follows the intravenous injection of fractions of exudates is unlike the more slowly developing leukocytosis which follows intravenous injection of nucleic acid or sodium nucleinate, but does resemble the more rapidly developing leukocytosis which occurs after intravenous injection of nucleotides. Menkin [14] has reported similar observations in connection with studies of exudates in dogs and stated that the possibility is not precluded that inflammatory exudates may contain considerable quantities of nucleotides.

While a nucleotide has been isolated from human blood,[15] there have been no reports of determinations of the amounts of nucleic acid or its derivatives in the circulating blood in the presence of acute inflammation. We would expect an increase above a normal range if these substances were present at an area of acute inflammation and were carried in the circulating blood to effect leukocytosis. The present complexity of methods for the quanti-

tative analysis of these substances is one obstacle to be overcome in such a study of nuclear material. According to Sabin,[16] nuclear material derived from constant breakdown of tissue may regulate the normal number of polymorphonuclear leukocytes in the circulating blood.

The suggestion has been made that histamine or histamine-like substances, such as the hypothetical H-substance of Lewis,[17] may produce the polymorphonuclear neutrophilic leukocytosis accompanying acute inflammation. Moon, Lieber and Kennedy [18] reviewed earlier studies of the effects of histamine on leukocyte counts and found "inconclusive evidence" that a relationship existed between histamine and leukocytosis either in animals or in man. Following the intravenous injection of cats with histamine phosphate in doses of 1 to 2 mg., they regularly observed absolute polymorphonuclear neutrophilic leukocytoses within several hours after injection, with peaks 2 to 4 hours after injection and a return to normal leukocyte levels within 24 hours. However, Menkin,[14] who extracted histamine from exudates of dogs by the method of Barsoum and Gaddum,[19] reported that the intravenous injection of histamine in amounts equivalent to the amounts recovered from such exudates failed to induce leukocytosis in dogs. In another report, Moon [20] stated that sufficiently large amounts of histamine might be released from extensive areas of injured tissue to be a possible factor in the production of leukocytosis. However, he also considered that some substance, apparently not histamine, was released from injured cells and attracted leukocytes to an area of injury. Moon believed that this substance was perhaps also effective in the production of systemic leukocytosis.

Danzer [21] prepared isotonic saline extracts of various normal rabbit organs and tissues, including muscle, liver, testicle, brain and hemolyzed erythrocytes. He reported that the subcutaneous or intravenous injection into rabbits of 3 to 5 cc. of such extracts was followed in 5 hours by a marked polymorphonuclear (amphophil) leukocytosis, lasting several days to a week. Danzer considered that this leukocytosis was caused by proteins contained in the extracts.

Following the intracutaneous injection of hemolytic streptococci into rabbits, Nettleship [22] observed that absolute polymor-

phonuclear (amphophil) leukocytoses occurred within 1 hour and were maintained for 1 to 3 weeks. From correlation of the times of appearance of necrosis and leukocytosis, Nettleship postulated that substances released from the cytoplasm of necrosing polymorphonuclear leukocytes appeared to diffuse into the blood stream and cause the leukocytosis. He then prepared sterile water extracts of polymorphonuclear leukocytes of rabbits (amphophils) from 48-hour sterile aleuronat pleural exudates and from centrifugalized blood, and sterile water extracts of rabbit bone marrow. Nettleship [23] reported that the intravenous injection into rabbits of 4 to 10 cc. of such extracts was followed in 2 hours by marked polymorphonuclear (amphophil) leukocytoses which lasted several days. Nettleship considered that the leukocytosis-producing principle present in these extracts of hematopoietic tissue was probably protein in nature, and termed this substance "leucogen." He reported that the intravenous injection of sterile water extracts of nonhematopoietic rabbit tissues was not followed by such polymorphonuclear (amphophil) leukocytosis.

Menkin [24-26] produced pleural exudates of from 1 to several days' duration in dogs by the intrapleural injection of turpentine. He described the isolation from such exudates of two nitrogenous substances, which he termed "leukotaxine" and a "leukocytosis-promoting factor," both presumably liberated in areas of acute inflammation. Menkin considered "leukotaxine" to be a polypeptide which was capable of increasing capillary permeability and of inducing prompt polymorphonuclear leukocytic migration at the site of an injury. The "leukocytosis-promoting factor" has been described [14,26] as being associated with the globulins and capable of inducing absolute polymorphonuclear neutrophilic leukocytoses in dogs when injected intravascularly. Menkin stated that "it is highly improbable that the effect of exudates on the leukocyte level might in any way be referable to a direct action of turpentine *per se* contained in exudates." Exudative material from a burned area of skin on the leg of a dog was also reported to have been followed by leukocytosis when injected into the blood stream.[27]

Ponder and Macleod,[3] using intraperitoneal saline infusions, produced peritoneal exudates of approximately 18 hours' duration in rabbits. The intraperitoneal injection into another rabbit

of 10 cc. of supernatant fluid from such an exudate was reported to have been followed in 4 hours by a relative polymorphonuclear (amphophil) leukocytosis in the injected animal.

Our results seem to indicate that some substance or substances present in the supernatant fluid fractions of centrifugalized rabbit exudates of 5¾ to 12 hours' duration produced the neutrophilic leukocytoses observed in our experiments. The regular occurrence of the peaks of the neutrophilic leukocytoses 2 to 3 hours following the intravenous injection of supernatant fluid suggests that the same substance or substances may have produced the leukocytosis in all experiments.

The leukocytosis-producing property of these supernatant fluid fractions apparently did not depend on their concentrations of sodium chloride. Intravenous injections of equivalent amounts of sterile sodium chloride solutions, of the same sodium chloride concentrations as the supernatant fluids, into the same experimental animals failed to show neutrophilic leukocytoses when leukocyte counts were made during corresponding periods of the day.

Apparently bacteria and their products also may be eliminated as possible causes for the neutrophilic leukocytoses observed in these experiments, as repeated bacteriological studies yielded negative results.

At the present time we cannot make any statements regarding the physical and chemical nature of the leukocytosis-producing material or materials with which we are dealing in these experiments. Protein material undoubtedly is present in these supernatant fluid fractions, because clotting has occurred in those which have been kept for several days to several months.

Summary

Sterile peritoneal exudates were recovered 5¾ to 12 hours after the intraperitoneal injection into rabbits of sterile 0.9 per cent sodium chloride solution. These exudates were centrifugalized and their supernatant fluid fractions retained. Thirteen experiments were performed, in which 5 rabbits received 1 intravenous injection and 4 rabbits received 2 intravenous injections (at intervals of 58 to 106 days) of 5 cc. of sterile fresh supernatant fluid from these sterile peritoneal exudates.

Hourly leukocyte examinations in all 13 experiments showed that in each case a neutrophilic leukocytosis occurred a few hours after injection. In 5 of the 13 experiments there was an absolute neutrophilic leukocytosis. The peak of the neutrophilic leukocytosis occurred 2 or 3 hours after injection in 12 experiments and 4 hours after injection in the remaining experiment. There was a return to normal total and differential leukocyte counts 6 to 31 hours after the supernatant fluid injections.

Leukocyte examinations, made at hourly intervals during the same period of the day, of the same 9 rabbits without injection and of 5 of these 9 rabbits following intravenous injections of 5 cc. of sterile sodium chloride solutions, showed no neutrophilic leukocytosis in any experiment. The chloride concentrations of these sterile saline solutions were equivalent to the chloride concentrations of the supernatant fluids which were injected.

Conclusion

These results seem to indicate that some substance or substances present in the supernatant fluid fractions of centrifugalized saline-induced rabbit peritoneal exudates of 5¾ to 12 hours' duration can produce neutrophilic leukocytoses when injected intravenously into other rabbits.

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THE TREATMENT OF SPONTANEOUS BREAST ADENOCARCINOMAS IN MICE WITH EXTRACTS OF SPLEEN OR YEAST *

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INTRODUCTION

It is not possible, in a brief presentation of this subject, to review previous work done by others who have attempted to heal spontaneous malignant tumors in mice or other animals. Furthermore, such a review would present an almost uninterrupted series of failures extending over about 30 years unless radiotherapy was employed. Occasionally, complete regressions have been reported in a small group of animals. However, these scattered reports either dealt with a very small number of animals or lacked confirmation when the experiments were repeated.

Experiments with any new form of cancer treatment should be started on transplanted tumors, as large numbers of animals with tumors of this type are easily obtainable. After its therapeutic efficacy has been proven on transplanted tumors, the new procedure should be tested on spontaneous tumors, for if complete regressions are obtained in transplanted tumors the same therapy *may* show results in spontaneous tumors. However, if a therapeutic attempt fails in transplanted tumors, spontaneous tumors will not respond to that treatment.

Our work has followed this sequence. After Lewisohn [1] in 1938 had reported 60 per cent cures among 281 mice with sarcoma 180 with a concentrated splenic extract, extracts of spleen in a variety of preparations were tested in spontaneous breast adenocarcinomas in mice (Lewisohn, Leuchtenberger and Laszlo [2]). Maisin [3] reported that by adding baker's yeast to the food of his animals he was able to reduce the incidence of malignant tumors in mice which had been treated with benzpyrene.

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It seemed of considerable interest to test whether these tumor-growth-inhibiting properties of yeast could be made available in extract form and used successfully in spontaneous malignant tumors.

Classification of Extracts

For the sake of brevity we shall refer to the different extracts which were used in the treatment of spontaneous tumors in the following manner:

S1: Healed splenic extract prepared from spleens of mice previously healed of Sarcoma 180 by a concentrated splenic extract.

S2: Splenic extract purified by dialysis and fractionation from concentrated splenic extract.

S3: Splenic extract prepared by immediate cold extraction.

Y: Yeast extract.

Description of Extracts

Extract S1. Healed splenic extract was prepared in the following manner: The mice were killed with ether and their spleens were immediately removed, weighed, frozen on blocks of dry ice and ground in a mortar with sand. Distilled water and chloroform were added in the proportion of 50 cc. of water and 5 cc. of chloroform to 5 gm. of spleen. The mixture was shaken for about 8 hours in a shaking machine and kept in an icebox for at least 48 hours. The proteins were precipitated with 10 per cent trichloracetic acid. The mixture was centrifugalized for about 15 minutes at the rate of 3000 r. p. m. The supernatant, light yellowish fluid was passed through a filter paper and concentrated at 60° C. *in vacuo,* until 1 cc. of the extract represented 1 gm. of fresh spleen. The acid extract was neutralized with sodium hydroxide using litmus paper as an indicator, and filtered through a Seitz filter No. 3. Merthiolate (1:200,000) was added and the extract was collected in sterile ampules.

Extract S2. The concentrated spleen extract was treated by continuous dialysis *in vacuo* at reduced temperature (40° to 50° C.) in the apparatus of Hanke and Koessler [4] for a period of

48 hours. One hundred cc. of the extract was dialysed under a layer of toluene against 600 cc. of water which was changed three times. In pilot experiments it was found that the dialysate, after this treatment, was free of sodium chloride and failed to give a biuret reaction. Both the dialysate and the nondialysable residue were concentrated to the original volume *in vacuo* at temperatures below 60° C.

The dialysate was precipitated with absolute alcohol to give a final concentration of 95 per cent. Excessive concentration of the dialysate before alcohol precipitation should be avoided and we confined ourselves to a concentration of 40 per cent of the original volume. The precipitate was kept for 24 to 48 hours in an icebox, after which the precipitate was separated by decantation from the strongly yellow, alcoholic, supernatant fluid. The precipitate was washed several times with 95 per cent alcohol and centrifugalized. Finally it was dissolved in the smallest possible amount of water and again treated with alcohol as before. The combined precipitates were dissolved as completely as possible in water, the solution freed of alcohol *in vacuo* and the volume reduced to the volume of the original extract. The alcoholic supernatant fluids were collected, the alcohol removed *in vacuo*, and the residue brought again to the starting point.

Extract S3. Fresh, refrigerated spleens obtained from the slaughter-house were minced in trichloracetic acid and the suspension kept for 4 hours at temperatures between 0° and 4° C. with frequent agitation. The filtrate obtained was brought *in vacuo* below 60° C. to the desired concentration of 1 cc. per 1 gm. of fresh weight. The extract was neutralized before injection and in some instances the trichloracetic acid was extracted with ether before use.

Extract Y. Of fresh brewer's yeast, 2500 gm., corresponding to 500 gm. of dry substance, was washed several times with distilled water. The yeast was boiled for 7 minutes with 9000 cc. of distilled water to which 0.9 cc. of glacial acetic acid had been added. The extract was filtered through paper pulp and concentrated *in vacuo* at 70° C. to 1500 cc. It was precipitated with 1500 cc. of absolute alcohol. The filtrate was concentrated *in vacuo* at 70° C. to 900 cc.

Chemical and Pharmacological Data

Experiments, carried out in this laboratory during the past 1½ years, have dealt with the chemical and pharmacological aspects of the following problems:

1. *Characterization of Concentrated Spleen Extract.* The concentrated spleen extract contained 14 per cent total solids. The dry residue contained 16.1 per cent nitrogen and 9.3 per cent ash. The extract had a P_H of 6.2. The minimum lethal dose upon subcutaneous administration was 0.5 cc., corresponding to 70 mg. dry substance, for a 20 gm. mouse; and 0.35 cc., corresponding to 49 mg. dry substance, upon intravenous injection. This toxicity was at least partly caused by concomitant vaso-active substances, the presence of which is irrelevant for the tumor-inhibitory effect.

2. *Fractionation of the Concentrated Spleen Extracts.* Upon dialysis, the tumor-inhibitory activity was found in the dialysate. This was concentrated to about one half its volume *in vacuo,* then precipitated with twenty times its volume of absolute alcohol. The precipitate was washed repeatedly with 95 per cent alcohol and with absolute alcohol, and upon re-precipitation the activity remained in the precipitate. This precipitate corresponded to 15 to 20 per cent of the original solids. Its toxicity was reduced one third to one fourth as compared with the toxicity of the original extract. The vaso-active factors were largely eliminated and the tumor regression, caused by these purified extracts, ran its course without hemorrhagic phenomena.

3. *Extract from Fresh Spleen in the Cold.* Fresh calves' spleens, obtained in ice from the slaughter-house, were minced under ice-cold trichloracetic acid. The filtrate, 1 cc. of which corresponded to 1 gm. of spleen, contained the tumor-inhibitory principle. Therefore, under these precautions, the yield of the principle may be improved so as to make a subsequent concentration of the extract unnecessary.

4. *Yeast Extracts.* Aqueous yeast extracts proved tumor-inhibitory. The extracts were dialysed, precipitated with absolute alcohol, as in the case of the spleen extracts, and the precipitate purified, washed and re-precipitated as before. Activity was again found in the final alcohol precipitate. The results of chemical analysis and of toxicity tests are given in Table I.

TABLE I
Fractionation of Yeast Extract

Extract	Dry substance, per cent	N_2 in per cent of dry substance	P in per cent of dry substance		Reducing power of the extracts calculated as glucose (Hagedorn-Jensen) in per cent of the total solids			Growth-factor in mg.*	P_H	Toxicity (lethal dose) in cc. and mg. by intravenous administration
			Inorganic	Organic	Direct	After 0.04 normal NaOH	Difference			
Original crude extract	6.82	8.51	—	—	6.82	6.12	0.70	—	5.42	0.15 cc. = 10.20 mg.
Dialysate	5.15	8.16	—	—	7.12	6.16	0.94	—	5.90	0.20 cc. = 10.30 mg.
Alcohol soluble fraction	1.95	12.72	0.10	1.50	13.66	13.66	0	0.122	5.02	0.45 cc. = 8.80 mg.
Precipitate	3.20	6.72	3.40	2.44	17.67‡	13.47	4.20	0.023	6.60	1.10 cc. = 16.50 mg.

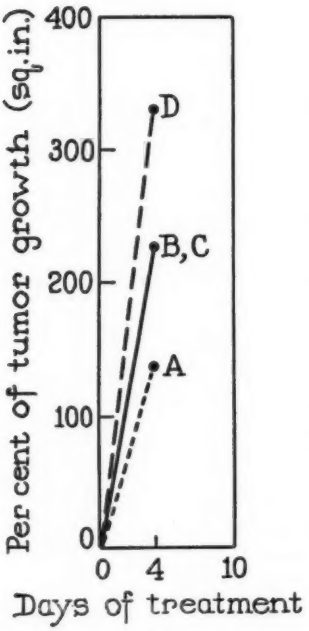
* Growth-factor = The smallest amount of extract in mg. of solids which supports the growth of *Bacillus influenzae* in autoclaved Difco medium.
‡ Reducing power after acid hydrolysis, 25.07.

The digestion of the precipitate with weak alkali on the boiling water bath destroyed part or all of the tumor activity, depending on the time of boiling and the concentration of alkali, and may even convert this activity into a tumor-growth-promoting factor.* The inhibitory activity disappeared, according to our experiments, in parallel with the disappearance of the ability of the same extracts to support the growth of bacteria on autoclaved media. The alcohol precipitate reduced alkaline potassium ferricyanide solution. This reducing power was partly destroyed by boiling with alkali, but the loss of tumor-inhibitory activity took place while the reducing power was still intact.

5. *Tumor Growth Curves as Preliminary Tests.* In a large number of transplanted tumors we have planimetrically measured the growth during a given period, and have compared area increment of extract-treated tumors with untreated controls. When using potent extracts we have found, even within the first week, a diminution of growth as compared with the controls. The amount of inhibition of growth may be used as an indicator for the potency of the extracts. This measure parallels, in many instances, the survival rate of the tumor-transplanted

* In recent experiments we have studied the effect of heat at various P_H levels on the growth-inhibiting properties of yeast extracts.

animals (Text-Figure 1). We are now engaged in the statistical treatment of this aspect of the problem.



TEXT-FIGURE 1. Effect of different extracts on tumor growth after 4 days of treatment. C = control. A = 1.5 per cent solution of the alcohol insoluble fraction. B = 1.5 per cent solution of the alcohol insoluble fraction boiled 3 minutes with alkali. D = 1.5 per cent solution of the alcohol insoluble fraction boiled 60 minutes with alkali.

TECHNIC

The technic (route, dose, frequency) of the injections has been discussed in detail in previous papers.[1,2] The spleen extracts were given intravenously or intraperitoneally. The yeast extract was given intravenously, usually combined with a subcutaneous injection.[5] The average dose for intravenous or intraperitoneal injection was 0.1 to 0.15 cc., whereas 0.5 cc. was given subcutaneously. Up to forty-three intravenous injections have been given to the same animal. Injections were given daily (in the majority of cases) or on alternate days.

BIOLOGICAL OBSERVATIONS

The material under consideration consisted of 363 mice with spontaneous breast tumors, in all of whom a biopsy was performed to establish the diagnosis. Whenever the slightest doubt existed as to the definitely malignant character of the tumor, the animal was excluded. Forty-one (11 per cent) proved to be nonmalignant. Additional proof that the diagnosis of malignancy was justified in the remaining 322 mice can be found in the fact that lung metastases were present in 30 per cent of those which came to autopsy. The great importance of a biopsy of spontaneous tumors is evident because it is impossible to establish the malignant or nonmalignant character of these tumors by palpation.

It is generally accepted that spontaneous tumors whose malignant nature is proven by biopsy do not disappear spontaneously. We felt that it was of great importance to establish whether biopsy alone might not influence the natural evolution of these tumors. No data on this question could be found in the literature. Sixty-eight tumor mice (60 from the Jackson Memorial Laboratory and 8 from the Rockland Farms) were used as controls, exposing them to biopsy only. Up to now we have not observed disappearance of the tumors in these controls, though some tumors showed temporary regression.

Twenty-eight animals were discarded for the following reasons: (1) death due to ether before biopsy was taken, (2) death during biopsy (in a number of instances pulmonary metastases were found at postmortem examination), (3) pregnancy, (4) cannibalism by other animals, (5) died without treatment or during the first 2 or 3 days.

Twenty-one animals are still under treatment. Sixteen other animals were treated with an immune spleen extract as described in a previous paper.[2] While 4 of these tumors disappeared temporarily, they all recurred. Therefore, these 16 cases are not considered in this report. Thus only 189 mice are the subjects of this presentation.

Among these 189 animals, 38 are healed at present. Of these, 27 were treated with various spleen extracts and 11 received yeast extract. There were 11 additional animals, treated with spleen extracts, in which a complete retrogression was first effected, but subsequently these animals showed recurrences (Table II). As stated previously,[2] we feel that these recurrences may be ex-

TABLE II
Summary of Apparently Healed Animals, of Recurrences and of Animals Remaining Healed

Extract	Animals apparently healed	Animals with recurrences	Animals remaining healed
Splenic S1	22	7	15
" S2	7	1	6
" S3	8	3	5
" R1	1	0	1
	38	11 (29%)	27
Yeast	11	0*	11

* Up to March 1, 1940. Oldest healed animal: 72 days since complete macroscopic regression.

plained by the fact that treatment was discontinued immediately after the tumor had disappeared. Since our procedure has been changed and treatment has been continued even after complete disappearance of the tumors, we have not observed recurrences. However, the final decision on this important point must await the natural death of the 38 healed animals which are living at present.

Among the 11 recurrent tumors, 10 occurred apparently in the original location, whereas 1 was observed in an entirely different part of the body. It is impossible to say whether these 10 new tumors occurred in exactly the same location or in an adjacent breast. The mammary glands in a mouse are in such close proximity that an exact evaluation of this point is practically impossible.

All of the recurrent tumors have been completely resistant to treatment. We have not been able to influence their growth, even when they were small. Thus, whereas large primary tumors responded to treatment, the recurrent tumors in the same animal (even when they were of small size) continued to grow in spite of frequent intravenous injections. Apparently the tumor cells have become resistant to the treatment.

It might appear from these statistics that the percentage of healed animals was only 20 per cent (38 among 189 animals). However, the actual figures do not give a correct picture. Working in a new field, we experimented with a large number of different extracts or their fractions. Some of these were complete failures. For instance, in a considerable number of mice, necrosis of the tail was noted after a few injections and further treatment had to be discontinued. Other fractions proved to be very toxic. Furthermore, a severe, intercurrent infection killed a considerable number of animals which were practically healed and whose tumors possibly would have disappeared completely.

When using effective and nontoxic extracts we maintained the percentage of total regressions at approximately 30 per cent.

In a previous paper [2] we mentioned that in addition to the healed animals with complete regressions, 30 per cent of the mice showed a reduction in the size of the tumors at the time of death. If we were to include these animals, we would arrive at a total figure of 60 per cent in which the tumor growth was influenced favorably. However, we prefer not to include this latter group.

We have, in a large number of cases, observed a temporary reduction in size during treatment with subsequent increase in growth. Thus we must not lose sight of the fact that these tumors might have started to grow again, if the animals had not succumbed. Furthermore, we have noted among the controls, marked transient reduction in the size of the tumors following simple biopsy. The only proper criterion is the complete and permanent disappearance of the tumor.

Of interest among the healed animals is the group of 5 cases (Table III) in which microscopic postmortem examination 149, 129, 93, 49 and 19 days after the mice were pronounced healed,

TABLE III
Number of Days Which Have Elapsed Since Tumors Were Healed
(Last Reading: March 1, 1940)

Animal No.	Extract	Days elapsed since complete regression of tumor	Animal No.	Extract	Days elapsed since complete regression of tumor
J.M. 210	S1	216	J.M. 362	Y	72
J.M. 199	S1	205	J.M. 350	Y	70
J.M. 228	S1	197	J.M. 310	Y	66
J.M. 207	S1	171	J.M. 307	S2	66
J.M. 153	S1	155 animal lost	J.M. 364	S1	65
J.M. 155	S2	149 no carcinoma*	J.M. 329	S2	55
J.M. 232	S3	133	J.M. 327	S2	51
J.M. 234	S3	133	J.M. 367	S1	49
J.M. 204	S3	132	J.M. 143	S1	49 no carcinoma*
J.M. 147	S1	129 no carcinoma*	J.M. 404	Y	44
J.M. 132	S1	120	J.M. 338	S3	41
J.M. 275	S1	108	J.M. 352	Y	40
J.M. 278	S3	108	R. 22	Y	39
J.M. 271	S1	108	R. 18	Y	38
J.M. 194	R1	104	R. 21	Y	35
J.M. 263	S1	102	J.M. 464	Y	26
J.M. 304	S2	102	J.M. 402	Y	23
J.M. 157	S2	93 no carcinoma*	J.M. 134	S1	19 no carcinoma*
J.M. 363	S1	81	R. 27	Y	12

Animals 134, 143, 147, 155, 157 died. Others are still living.
J.M.=Jackson Memorial Laboratory. R.=Rockland Farms.
R1=Concentrated spleen extract (100 gm. in 1 cc.) prepared in this laboratory and used in a few animals.
* As determined by postmortem microscopical examination.

failed to show a trace of cancer cells. They seem to offer additional proof that these cancers were healed by splenic extracts. The 11 animals healed with yeast extract (first treatment November 9, 1939) were all alive at the time of this report.

The fact that two different extracts (spleen and yeast) caused total regressions in these tumors, may help in the search for the active principle. We have started some investigations along these

lines. Nicotinamide is not tumor-active. Possibly cozymase may prove to be a tumor-reducing substance.

Summary

1. Thirty-eight spontaneous breast adenocarcinomas in mice, proven by biopsies, disappeared completely following treatment with spleen or yeast extracts.

2. The method of preparation of the different extracts is presented.

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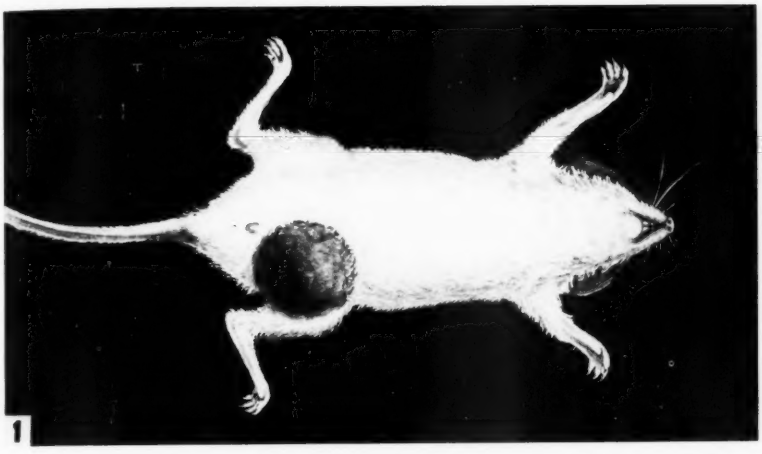
DESCRIPTION OF PLATES

Plate 48

Fig. 1. Mouse No. J.M. 404, healed by yeast extract. Lesion was proved to be carcinoma by microscopical examination. Treatment was started on December 23, 1939 and growth had disappeared by January 16, 1940. This animal received 12 intravenous and 10 subcutaneous injections of yeast extract.

Fig. 2. Mouse No. J.M. 402, healed by yeast extract. Neoplasm was proved to be carcinoma by microscopical examination. Treatment was started on December 23, 1939 and growth had disappeared by January 16, 1940. This animal received 11 intravenous and 10 subcutaneous injections of yeast extract.

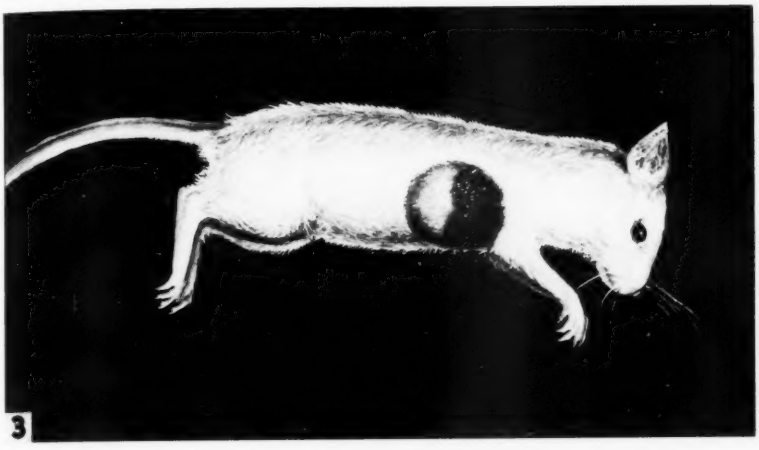


Lewisohn and Associates Treatment of Adenocarcinomas in Mice

PLATE 49

FIG. 3. Mouse No. R. 27, healed by yeast extract. Neoplasm was proved to be carcinoma by microscopical examination. Treatment was started February 5, 1940 and growth had disappeared by February 17, 1940. This animal received 8 intravenous and 7 subcutaneous injections of yeast extract.

FIG. 4. Control mouse No. R. 8, proved to have carcinoma by biopsy. Drawings to show progress of growth in this untreated animal between January 3, 1940 and February 23, 1940.



Lewisohn and Associates Treatment of Adenocarcinomas in Mice

PLATE 50

FIG. 5. Control mouse No. R. 11. proved to have carcinoma by biopsy. Drawings to show progress of growth in this untreated animal between January 3, 1940 and February 23, 1940.

Lewisohn and Associates Treatment of Adenocarcinomas in Mice

UNIV. OF MICH.

PRIMARY FIBROMYXOSARCOMAS OF THE HEART AND PULMONARY ARTERY *

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Primary myxomas, fibromyxomas and fibromyxosarcomas of the heart have been recognized for many years, but they are still very rare. According to Diebold,[1] the first case was reported by von Albers in 1835, a citation which we were unable to verify. In the postmortem service of the Allegheny General Hospital we recently had a case of primary fibromyxosarcoma originating in the first portion of the pulmonary artery, extending along the pulmonary tree and producing multiple metastases in the left lung and bronchi and in the vessels of the right lung. We believe the case to be of general interest not only because it is unusual, but because the site of origin and the manner of extension appear to throw some light on the histogenesis of tumors of this type.

Fibromyxosarcomas of the heart are far less common than the myxomas from which they apparently develop. The literature dealing with cardiac myxomas contains numerous discussions concerning their true nature.

Thorel, in a series of articles on tumors of the heart published in 1903,[2] 1907,[3] 1910[4] and 1915,[5] assembled reports of 24 cases that had been reported as fibromyxomas of the heart chambers. He maintained that they were organized thrombi and stated in his final report that, with the single exception of the case of Horneffer and Gautier,[6] which had metastasized, all of the tumors were explainable on the basis of the organization of a thrombus. Among the cases cited by Thorel was that of Kesserling,[7] who advocated the theory that fibromyxomas of the heart valves arose in embryonic rests associated with the anlagen of the heart valves, admitted to be of myxomatous tissue, and that of Bacmeister,[8]

* Received for publication August 1, 1940.

who seems to have been the first to postulate that the tumors began as organizing thrombi and underwent metaplasia into true neoplastic growths. Thorel quoted Czapek [9] in support of the organizing-thrombus theory and concluded finally that, if the structures were true tumors, there were two possibilities for their explanation: (1) they arose from myxomatous metaplasia of a thrombus; and (2) they came from the myxomatous anlagen of the valves, rests of which either might persist in accordance with Ribbert's theory or revert in later life to an embryonic myxomatous state.

Brenner [10] in 1907 collected 32 myxomas of the heart and added 1. Of these, 19 were in the left auricle, 10 on heart valves, 2 in the right ventricle and 1 beneath the epicardium at the apex. Of the 19 in the left auricle, 8 were on the septum, 6 above the mitral valve and 5 at various other points on the wall. Two of those which occurred on valves were on the mitral, 6 on the tricuspid and 2 on the pulmonic. Brenner believed the tumors to be true neoplasms, although he said they could not all be differentiated in the gross but had to be diagnosed microscopically; this could be done on their arrangement and cytology, and sometimes microchemically by the use of the mucicarmine reaction. Husten [11] in 1922 collected 71 polypoid tumors of the endocardium of the right heart and 17 of the left. He divided and classified them into organized thrombi, pseudotumors and true myxomas. He accepted only 9 from the right heart and 2 from the left as true myxomas. He cited six authors who had reported sarcomas but did not discuss them, since they were definitely malignant. Kirch [12] in 1927 added 12 myxomas, including 2 hemangiomyxomas, and concluded that the older views of Thorel and Czapek could no longer be held but that the growths were true neoplasms.

Other cases of polypoid tumors of the heart which we were able to review and which resembled ours in the gross included those of Binder,[13] 1913 (sarcoma); Binder,[14] 1927 (myxoma); Barnes and Yater,[15] 1929; Jaleski,[16] 1934; Fossel,[17] 1936, 2 cases; and Müller,[18] 1932, who reported 3 myxomas and 1 sarcoma.

Diebold [1] in 1930 was the first to separate the sarcomas of the heart from the myxomas. He collected 45 cases and prepared a table giving the authors, sites of the primaries and metastases, microscopic diagnoses and clinical notes. Of 34 cases upon which

the data were complete, 25 were males, 9 were females and all were between the ages of 20 and 70 years. The primaries occurred in the right auricle 14 times; right ventricle, 3; pulmonary artery, 1; left auricle, 8; left ventricle, 1; auricular septum, 1; and ventricular septum, 1. Twelve cases produced metastases which occurred in the lungs 6 times; kidney, 3; lymph nodes, 3; and once each in the heart itself, pericardium, pleura, liver, pancreas and brain. The microscopic diagnoses included 15 spindle cell sarcomas, 12 round cell sarcomas, 4 giant cell sarcomas, 4 myosarcomas and 3 mixed cell sarcomas. Diebold's own case was diagnosed a "mixed cell" sarcoma, but both his description and his pictures resemble our own case closely and we believe it could have been interpreted as a fibromyxosarcoma. The only case beside our own in which the primary site was given in the pulmonary artery was that of Esbach, quoted by Diebold.

Since Diebold's [1] report we have found 2 additional cases with metastases. Müller [18] in 1932 described a case of primary "fibrosarcoma" of the left auricular wall with secondary nodules of the lungs and pleura. Both his pictures and his microscopic description lead us to believe that his neoplasm was a fibromyxosarcoma similar to ours. Fenster [19] in 1933 reported a "malignant myxoma" of the right auricle with multiple metastases to the liver, retroperitoneal lymph nodes, kidneys, adrenals and brain.

Yater [20] in 1931, in a review of all tumors of the heart and pericardium, found a total of 49 primary sarcomas of the heart. Of these, 15 had produced metastases and the diagnosis of myxosarcoma had been made in only 3 instances. Pollia and Gogol [21] in 1936 reviewed the incidence of heart tumors in 46,072 autopsies and found 154 primary and 220 secondary neoplasms.

As far as we have been able to ascertain, our case is the second fibromyxosarcoma occurring in the pulmonary artery.

Report of Case

Diagnoses. Primary fibromyxosarcoma at root of pulmonary artery, extensive metastases to left lung and to pulmonary arteries of right lung, pulmonary arteritis, multiple hemorrhagic infarcts of right lung, hydrothorax, edema of ankles, benign leiomyoma of uterus, and thrombosis of uterine venous plexus.

Clinical History

The patient,* A. K., was a white housewife, age 51.

Eight months before admission to the hospital the patient noticed pain about the heart and shortness of breath. She was placed at rest in bed with a diagnosis of pleuritis and, after 1 month, was apparently cured. Later the shortness of breath recurred and gradually increased. About 2 months before admission the patient suffered an attack of hemoptysis, which was not repeated. There were several gastro-intestinal upsets not accompanied by bloody stools. The patient had lost weight, although she was still rather stout. Her past history, as well as the family history, was irrelevant.

Physical Examination. The patient was well nourished and well developed. She exhibited severe respiratory distress. The skin was of normal color, and there were no palpable nodes. Eyes, ears, nose and mouth appeared normal. The chest was symmetrical, although there was limited motion on the left side, especially in the upper portion. Breath sounds, vocal fremitus and tactile fremitus were decreased over the upper, anterior portion of the chest, and no râles or rhonchi were present. There was a healed cholecystectomy scar in the right upper quadrant. Temperature, 100° F.; pulse, 98; respirations, 24; blood pressure, 110/72 on admission.

Laboratory Examinations. Laboratory findings showed the urine negative; a slight anemia; r. b. c., 4,040,000; w. b. c., 11,500; Hb., 69 per cent. Differential counts gave an excess of polymorphonuclear leukocytes up to 93 per cent. An autopsy was performed on December 2, 1939, 4 hours after death.

Roentgenologic Report. The first films were made on March 17, 1939. At that time there was a rather dense shadow at the base of the left lung and evidence of an enlarged gland in the root of the left lung. Films, made on August 9, revealed cloudiness in the lower two thirds of the left lung. There were three large tumor shadows. Those made September 26 showed that the tumor shadows had enlarged so that they were probably twice the size found at the preceding examination. On October 3 a probable diagnosis of carcinoma of the left lung was made and the patient referred for X-ray treatment. The Department of Roentgenology considered the case one of Hodgkin's disease and treated the patient on that basis. There was rapid symptomatic improvement but films made on October 19 failed to show any decrease in the size of the tumor shadows. If anything, they were slightly larger. Treatment was continued until November 8, during which time 6090 r. were given, with about 2000 r. over each of the three shadows. Following the treatment on November 8, the chest filled rapidly with fluid. On November 10 a part of the fluid was removed and air was introduced. The tumor shadows at that time had not changed in size or appearance.

Synopsis of Postmortem Findings

The body was that of a middle-aged, white female with extensive edema of the subcutaneous tissues of the chest and of both ankles.

The left pleural cavity contained about 1000 cc. of clear, straw-

* On the service of Dr. W. B. Ray and referred by Dr. William Marshall, of Aspinwall, Pa.

colored fluid and the right about 500 cc. The left lung was attached by dense, fibrous bands covering all of the pleural surfaces except the diaphragmatic. The right lung was adherent at the apex and over the posterior portion of the heart.

Heart. The pericardial sac contained 200 cc. of clear fluid. *In situ* the heart was distended. The right auricle was greatly dilated. The apex was situated beneath the fifth interspace in the axillary line and the base extended to the right beyond the sternal margin. When empty the heart weighed 400 gm. and measured 14 by 11 by 5 cm. The epicardium was smooth and glistening. The subepicardial fat was excessive, and watery in appearance. The myocardium was flabby and dark red. The auricular and ventricular cavities were normal. The pulmonary valve orifice was enlarged and the pulmonary artery was increased in diameter. All other valvular orifices were of average size and all valve cusps were pliable and apparently in good condition. The left ventricular wall was 1.3 cm. in thickness.

In the pulmonary artery three myxomatous polyps were attached to the posterior wall at a point about 3 cm. above the corpus arantii of the middle semilunar cusp. The polyps hung downward in the pulmonary artery and almost occluded it. They were grayish in color and were of aspic-gelatin appearance and consistency. Their pedicles were united in a common, broad base. The largest polyp was roughly pear shaped and measured 3 cm. in length and 2.5 cm. in its greatest diameter. It was pendulous and hung freely over the other two polyps and slightly to the right of the next smaller one. This one was egg shaped and had a very short pedicle. It measured about 2.5 cm. in all diameters. The smallest polyp measured 1.5 cm. in length by 0.6 cm. in diameter and was completely obscured until the largest polyp was lifted. The surfaces of all the polyps were smooth, glistening and semitransparent. For at least 2 cm. to the left, 1 cm. above and below and 3 cm. to the right of the base of the polyp the arterial wall was thickened, reddened, appeared ulcerated and was covered with a layer of fibrin of varying thickness. Directly over the center of the right semilunar cusp there was a thickened nodule about 8 mm. in diameter which protruded for a distance of 4 mm. into the lumen. It was hemorrhagic but appeared to be solid throughout. About 1 cm. above the right semilunar valve

there was a second smaller nodule, not more than 3 mm. in diameter, which was red in color.

Aorta. The aorta showed slight yellowish streaking. Small atheromatous plaques and ulcerations were present in the thoracic and abdominal portions. None was calcified.

Left Lung. The left lung weighed 750 gm. and measured 20 by 11 by 6 cm. The pleura was covered with broken adhesive bands. When placed in water the lung sank except for a portion of the lower lobe. A soft, tenacious, gelatinous, tan-colored polyp protruded from the main bronchus. On section the central portion of the lung was replaced by new growths made up of nodular, gelatinous masses. At least two thirds of the lung was involved. The nodules varied from 1 to 8 cm. in diameter. Some were cystic and others contained hemorrhagic zones. Some were located in veins which they distended, some were in bronchi and others were nonencapsulated and grew freely in the parenchyma. The intravascular extensions grew as elongated polyps or resembled obturating thrombi undergoing white softening. Beneath the pleura the remaining lung was in part air-containing and in part collapsed from pressure of the tumor and the pleural effusion. Moderate anthracotic pigmentation was present.

Right Lung. The right lung weighed 450 gm. and measured 22 by 14 by 4 cm. Most of the lung was air-containing. Several of the smaller pulmonary arteries were filled with gelatinous growths. Near the apex there was a recent wedge-shaped, hemorrhagic infarct. In the lower lobe there was a grayish brown, infarcted area undergoing organization.

Uterus. The only abdominal organ that presented a gross pathologic lesion was the uterus. It was somewhat enlarged and the cavity was distorted by the presence of a fibroid tumor, 6 cm. in diameter, located in the posterior wall. At the margin of the growth there were several dilated veins of the uterine plexus, filled with thrombi. Most of these were deep red in color, although some appeared to be well organized and of a red-brown color.

Microscopic Examination

Heart. The cardiac muscle cells were of normal size and staining qualities.

Polyps of Pulmonary Artery. A striking feature about the polyps was the great excess of myxomatous stroma in comparison to the cellular elements. The external surfaces were smooth and probably had been covered with endothelium. There was a concentration of the stroma just beneath the surface and this zone was more cellular than the central part of the polyp. At the junction of the polyp with the pulmonary arterial wall there was an interchange of cells and vessels with those of the pulmonary artery. The most cellular part of the growth was near the attachment. Here the cells were very numerous and varied greatly in size and shape. Some of the cells were spindle shaped with long, stellate processes, and others were round or oval. Many of the cells were multinucleated and appeared to be true neoplastic forms. The multinucleated cells had from 2 to 30 or more nuclei. Some of them resembled striped muscle cells but could not be shown by special methods to have cross striae. In addition to the tumor cells there were numerous single and multinucleated phagocytes, occasional lymphocytes and numerous red cells. Mitoses were numerous in some zones. In the more cellular zones there was a fair amount of collagen in the stroma. As the central portions of the polyp were approached there were fewer tumor cells and many of those that were present were unstained. Near the central portion the stroma was very edematous; large, cystic spaces were present, and numerous hemorrhagic areas were seen. The number of vessels varied and most of them were capillaries or thin-walled arterioles. The sessile nodule of the pulmonary artery proved to be a deep-seated, hemorrhagic, atheromatous cyst.

Pulmonary Artery Near Attachment of Polyp. The intima was ulcerated and covered with adherent, hyalinized fibrin undergoing organization. This zone contained many mononuclear phagocytes and less numerous multinucleated giant cells. Beneath the ulcerated surface the intima and part of the media presented a myxomatous appearance and were infiltrated with typical neoplastic cells of the type already described.

Aorta. The aorta showed a moderate degree of intimal sclerosis.

Lungs. A section of the main bronchus showed a very active inflammatory process. The lumen was filled with a myxomatous polyp of exactly the same structure as that found in the pulmon-

ary artery except that it was unattached at the point where the section had been taken. Other sections containing large branches of the pulmonary artery showed them to be filled with neoplastic thrombi. Some of these completely closed the vessel and infiltrated the walls, while others were mural with finger-like polypi extending out of the main masses. One vessel showed several points at which the tumor was penetrating its walls and invading the lung. At the points of perforation the cells were growing at right angles to the vessel wall. The secondary nodules in the lung tissue showed concentration of the cellular and fibrous elements in their peripheries but appeared to displace and compress the surrounding lung tissue rather than to form definite capsules. In the right lung there were two areas of hemorrhagic infarction, one of which was partially organized. Vessels leading into these areas were filled with simple emboli without tumor cells. It was thought that these originated from the thrombi in the uterine plexus.

Uterus. The tumor of the uterus was a very dense leiomyoma of the fibroid type in which multiple sections failed to show any unusual growth activity.

Discussion

The neoplasm in this case was a malignant tumor originating at the root of the pulmonary artery and belonging in the category of myxomas, fibromyxomas and fibromyxosarcomas of the endocardium.

Microscopically it could have been called a spindle cell sarcoma, a mixed cell sarcoma or even a giant cell sarcoma if cellular morphology alone had been considered. The diagnosis of fibromyxosarcoma was made on the presence of large, spongy, round and spindle-shaped cells with stellate fibrils spreading in the loose meshes of the stroma, and on the nature of the stroma itself. The cells varied greatly in size and in the number of nuclei. Many were in mitosis. The stroma was typically myxomatous with peripheral zones of collagenous fibrils. It was watery in appearance and tended to undergo necrosis and form cysts. The type of stroma offered no support to the proliferating capillaries and areas of hemorrhage were common. The metastatic growths established the diagnosis of sarcoma.

As to the origin of polypoid tumors of the heart, the views of

Thorel, Czapek and others that they are organized thrombi could be excluded at once in our case. The location of the tumors on the wall of the pulmonary artery at some distance from any point at which myxomatous anlagen are known to exist makes it extremely unlikely that they arose in an embryonic rest. The interpretation of Bacmeister and others that these tumors are derived by myxomatous metaplasia within a thrombus could not be excluded. In view of the facts that there was an ulcerated area of the pulmonary arterial wall covered by a hyaline, mural thrombus in the immediate vicinity of the pedicle, that there was no line of separation between the arterial wall and the elements of the tumors, that the blood supply of the new growth was continuous with vasa vasorum and that fibroblasts, tumor cells and capillary endothelium were common to the growth and to the arterial wall and were generously intermingled, we feel that organization and metaplasia offer the most logical explanation. It is no more difficult to accept the possibility of neoplastic metaplasia in a zone undergoing reparative proliferation than it is to accept the abrupt establishment of a malignant growth in otherwise apparently normal tissues.

Conclusions

1. An example of fibromyxosarcoma of the pulmonary artery is described. This is probably the second tumor of its type to be reported in this location.

2. The primary growths resembled organized thrombi in the gross, yet presented all of the microscopic evidences of malignancy.

3. There were extensive metastases along the pulmonary arterial tree and in the lungs and bronchi.

4. There was a zone of pulmonary arteritis, about the pedicle of the tumor, covered with an organizing thrombus that showed no neoplastic changes.

5. The association of pulmonary arteritis with the base of the tumor suggests that a logical sequence in the tumor's development may have been: pulmonary arteritis with loss of endothelium, protective thrombosis, organization of the thrombus with myxomatous metaplasia, sarcomatous change in the myxoma and secondary extensions to the lungs by way of the branches of the pulmonary artery.

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DESCRIPTION OF PLATES

PLATE 51

FIG. 1. Gross photograph of the heart showing the attachment of one small and two large polyps to the wall of the pulmonary artery 3 cm. above the middle semilunar cusp. Note the thickening and roughening of the pulmonary artery.

1

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PLATE 52

FIG. 2. Gross photograph of left lung showing extensive secondary myxomatous tumors. In the center a polypoid mass may be seen protruding from the main bronchus. The large, bulbous structure in the lower right-hand corner was a cystic, polypoid mass which was squeezed out of the lung when it was sectioned.

2

PLATE 53

FIG. 3. Low power photomicrograph through the smallest of the three polyps and showing the continuity of the growth with the pulmonary arterial wall. The cellular areas of the tumor were just beneath the surface and along the line of attachment. × 22.

FIG. 4. Wall of the pulmonary artery at a point near the attachment of the polyp. Note the arteritis, ulceration and deposit of hyaline fibrin on the surface. × 30.

3

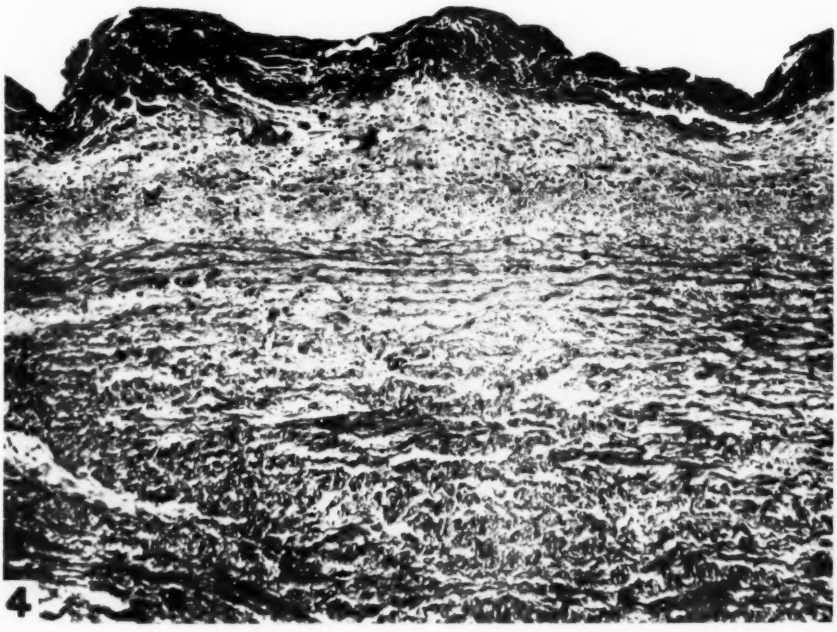
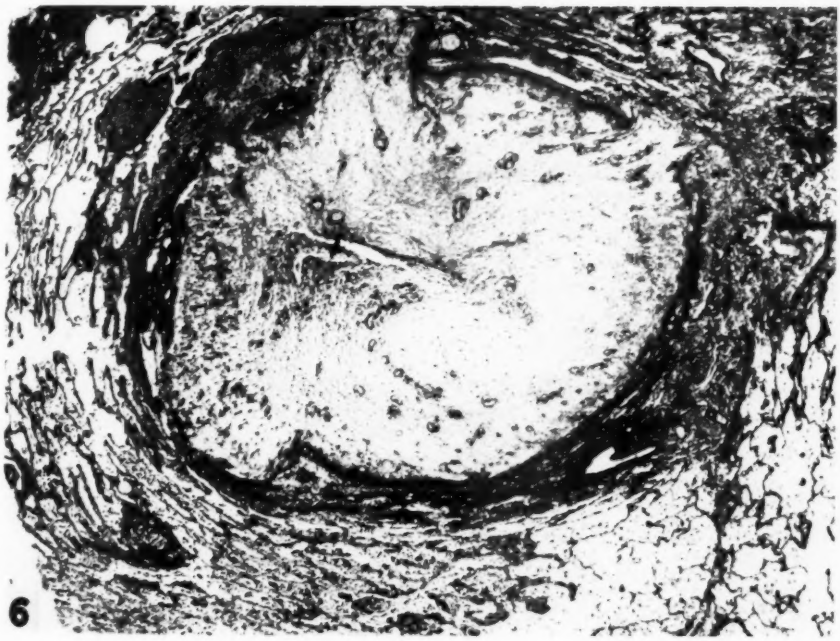

4

PLATE 54

FIG. 5. Section of the pulmonary artery containing a secondary myxomatous growth. The vessel is, for the most part, occluded, although parts of the tumor are growing as long, filamentous polyps. × 8.5.

FIG. 6. A small branch of a pulmonary artery, the wall of which was penetrated at several points by direct extensions of the tumor into the parenchyma of the lung. × 30.

5



6

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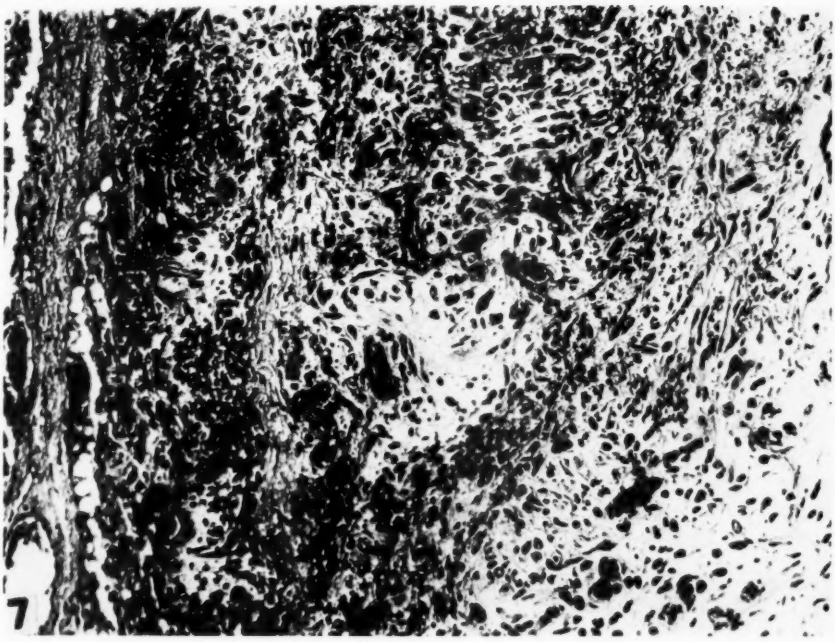
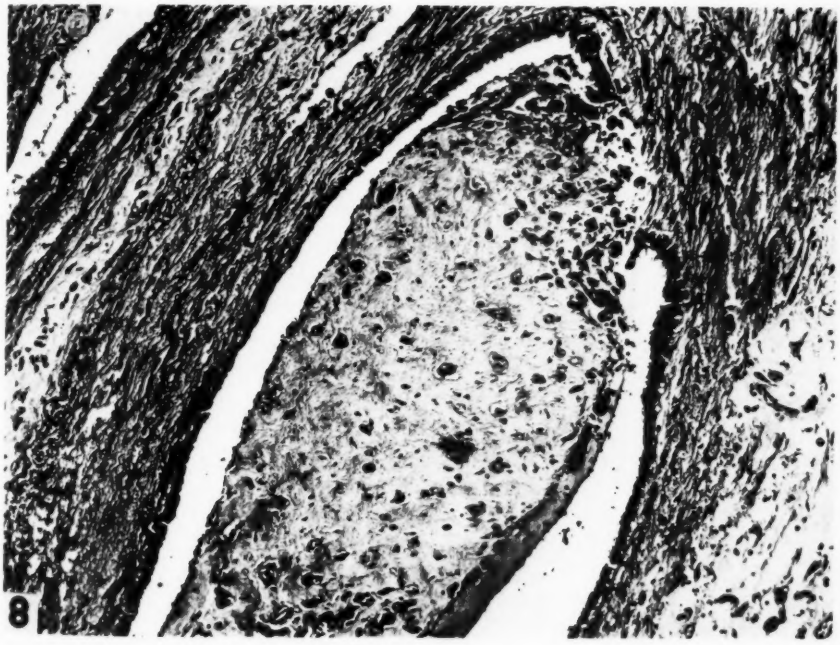
PLATE 55

FIG. 7. High power photomicrograph of one of the secondary tumors of the lung, showing a great variation in the shape and size of the tumor cells and many multinucleated forms. × 180.

FIG. 8. High power photomicrograph of a secondary polyp extending through the wall and growing freely in a small bronchiole. × 180.





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UNIV. OF MICH.

BACTERIAL LOCALIZATION IN THE KIDNEYS *

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Evidence as to the rôle of the kidneys in the removal of bacteria from the blood and their excretion into the urine is conflicting. Although microörganisms not infrequently enter the blood stream and pass through the vascular bed of the kidneys, there is still uncertainty as to the extent to which normal kidneys can remove them from the blood and destroy them or excrete them through the urine. Some workers have concluded that normal kidneys can excrete bacteria (Biedl and Kraus,[1] Schweizer,[2] Rolly,[3] MacKenzie and Cochrane [4] and Cohnheim [5]) while others have come to opposite conclusions (Pernice and Scagliosi,[6] Sherrington,[7] Asch,[8] Matsuyama,[9] Dyke,[10] Book,[11] Helmholz and Millikin [12] and Kirkpatrick [13]).

These conflicting opinions are probably due to differences in experimental procedure depending upon the number of bacteria injected, conditions and time intervals under which the urine was collected and cultured, and the presence of unsuspected renal lesions. They might also be due to the production of mechanical or other injury to the kidneys during the course of the experiments. In some instances in which bacteria were injected intravenously or into the renal artery, glomerular lesions could be demonstrated. Book,[11] for example, injected living staphylococci intravenously into rabbits and was unable to find them in the urine until about 12 hours later. Large numbers, in fact, did not appear until after 18 to 24 hours. When he injected colon bacilli, the organisms were not found in the urine until 48 hours had elapsed. Histological studies, made by removing the kidneys and incubating them at 37° C. in order to allow the bacteria in the kidneys at the time of removal to develop into colonies, showed the bacteria, in most instances, in capillaries of the cortex and medulla. Only in animals in which growth into the glomeruli and

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into Bowman's space had occurred were microörganisms cultured from the urine.

We decided to perform experiments, using particularly the method employed by Book,[11] attempting to determine to what extent normal kidneys can excrete living bacteria, in what ways the kidney can remove living bacteria and other particulate materials from the circulating blood, and the localizing potentialities of the kidney for such particles, both in normal organs and in those in which acute experimental hydronephrosis had been produced.

Materials and Methods

Adult male rabbits were used. The problem of excretion of living bacteria into the urine was studied by injecting 24-hour broth cultures of *Staphylococcus aureus, Bacterium coli* or *Bacillus typhosus* intravenously into the rabbits. These animals had previously been anesthetized with nembutal and their bladders exposed by low laparotomy. At intervals of from 5 to 120 minutes, samples of urine were withdrawn from the bladder with a sterile syringe after searing the surface of the bladder with a red-hot spatula. Duplicate samples of 1 cc. each were mixed well with warm, melted agar culture medium and incubated at 37° C. for 48 hours.

The second procedure was to determine the influence of experimental hydronephrosis upon bacterial localization in the kidneys. Hydronephrosis was produced in the left kidney of each rabbit by ligating the ureter after either a mesial incision and transperitoneal approach or a left flank incision and extraperitoneal approach. In some rabbits we produced only a partial obstruction by placing an 18 gauge hypodermic needle under the ligature, with removal of the needle after the ligature had been tied. The injections were made 72 hours later, at which time acute hydronephrosis was well developed.

The influence of such hydronephrosis upon the ability of the kidney to remove particulate materials from the blood was studied in two ways. (1) Rabbits were injected intravenously with India ink (Higgins's White Label or Pelican) diluted 1:4, 1:10, or 1:20 with sterile normal salt solution. Seventeen animals were killed at varying periods after injection and liver, spleen and both kid-

neys were removed. Large transverse sections from the kidneys and also other tissues were stained with hematoxylin and eosin. (2) A 24-hour broth culture of either *Staph. aureus* or *Bact. coli* in 1 or 2 cc. portions was injected intravenously. At various intervals the rabbits were sacrificed. The kidneys were removed aseptically, placed in sterile Petri dishes and incubated at 37° C. for 24 hours in order to allow any bacteria present at the time of removal to develop colonies. After incubation the organs were fixed in Zenker-formaldehyde solution and microscopical sections stained by the Claudius modification of Gram's method.

By these methods one can compare the two kidneys exposed simultaneously to particulate materials in the blood stream and can observe the extent to which the mechanical effects of increased intrapelvic pressure may modify the blood flow and thus influence bacterial localization in the hydronephrotic kidney. Sections reveal where the particles are deposited in the kidney and thus help to disclose the mechanism influencing their deposition.

Results

Experiment I. The Excretion of Bacteria into the Urine after Intravenous Injection

Six rabbits were injected intravenously: 1 with *Staph. aureus;* 2 with *Bact. coli;* and 3 with *B. typhosus*. The results showed that in no instance did any of the injected bacteria appear in the agar plates from the urine samples, indicating that under these conditions the normal kidneys did not excrete bacteria in significant numbers during the period of the experiments (Table I).

Experiment II. The Effects of Experimental Hydronephrosis upon the Removal of Particulate Materials by the Kidney

Our first studies in this experiment were concerned with the distribution of carbon particles in the kidneys of rabbits with a left hydronephrosis of 72 hours' duration.

On gross examination at autopsy, performed from 3 minutes to 25 hours after injection, the black staining in the spleen, omentum and, in some cases, the liver and lungs was intense. The hydronephrotic kidneys in all cases weighed about twice as much as the normal kidneys. The pelves were dilated in all cases and each contained about 3 cc. of urine. This urine, in a few in-

TABLE I
Excretion of Bacteria in the Urine after Intravenous Injection into Normal Rabbits

Animal No.	Organism	Time elapsed	Urine culture (2 plates)	
		minutes		
1.	*Staph. aureus*	5	no growth	no growth
2.	*Bact. coli*	5	no growth	contam. staph.
		15	no growth	no growth
3.	*Bact. coli*	15	no growth	contam. *B. subtilis*
4.	*B. typhosus*	5	no growth	no growth
		20	no growth	no growth
5.	*B. typhosus*	8	no growth	no growth
		30	no growth	no growth
6.	*B. typhosus*	5	no growth	no growth
		20	no growth	no growth
		50	no growth	no growth
		70	contam. *B. subtilis*	no growth
		120	no growth	no growth

TABLE II
Localization of India Ink in Normal and Hydronephrotic Kidneys after Intravenous Injection

Injection	Animal No.	Time	Normal	Hydronephrotic
10 cc. of 1:4 ink	3	3 min.	+	+++
	4	7 min.	+	++
	20*	3 hrs.	+	++++
	18*	23 hrs.	trace	+++
	16	24 hrs.	+	++
	26*	25 hrs.	trace	++
20 to 40 cc. of 1:4 ink	22	4 hrs.	++++	++++
	23	5 hrs.	++++	++++
	24*	5 hrs.	+++	++++
	21†	25 hrs.	+++	++++
20 to 40 cc. of 1:10 ink	28*‡	12 hrs.	+	++
	7	22 hrs.	+	++
	27*‡	24 hrs.	trace	+++
	25	24 hrs.	+	++
20 cc. of 1:20 ink	6*	14 hrs.	trace	+
	5	24 hrs.	trace	trace
20 cc. of 1:40 ink	29*‡	12 hrs.	+++	+++

* Intracellular carbon was seen in the hydronephrotic kidneys. This, in most cases, was in large phagocytic cells in the areolar tissue of the pelvis.

† The right kidney in this rabbit displayed both gross and microscopic evidence of a previous inflammatory process. Both extracellular and intracellular carbon particles were seen in large amounts.

‡ These animals received intravenous injections of Pelican ink. The others received Higgins's White Label.

stances, was stained by the India ink, especially in those animals that had received relatively large amounts of ink. On cut section one could distinguish a grayish cast to the renal tissue, especially of the hydronephrotic kidneys. Otherwise there was little difference in the gross appearance of the normal and hydronephrotic kidneys.

The results of the histological examination of the kidneys of these rabbits injected with India ink are tabulated in Table II. In general, most of the carbon particles were present in masses in the glomerular capillaries, in greater amounts in the hydronephrotic than in the normal kidneys. The amount of carbon in a given section is expressed in terms of from 1 plus to 4 plus, depending upon the number of carbon particles per low power field. In most instances, some of the carbon particles had passed through the glomerular capillaries and had accumulated in the intertubular blood vessels. In the medulla, in addition to the carbon in the blood vessels, small granules or clumps were seen in the tissue spaces between dilated tubules. Although particles were seen in this location in both normal and hydronephrotic kidneys, they were present in much greater numbers in the latter. In the pelves of the hydronephrotic kidneys, carbon particles frequently lodged either singly or in clumps just beneath the pelvic epithelium in the areolar tissue. This was not so common or so marked in the normal kidneys.

In several rabbits carbon particles were seen within fixed phagocytic cells of the peripelvic areolar tissue, especially beneath the epithelium. In 2 rabbits carbon particles were present within large mononuclear cells located interstitially in the medulla. These intracellular carbon particles were seen only in the hydronephrotic kidneys. In 1 animal (No. 21) the right kidney, both grossly and microscopically, showed definite signs of a previous inflammatory process and in it there was much more carbon, both intracellularly and extracellularly, than in any of the other animals.

The gross appearance of the kidneys and other viscera of 29 rabbits that had received intravenous injections of bacterial cultures was not striking. The hydronephrotic kidneys again were found to weigh about twice as much as the normal kidneys. The dilated pelves contained an average of 3 cc. of urine, which in a

few cases appeared to be purulent. It was not cultured. No gross abscesses were seen in kidneys or other viscera.

A quantitative estimation of the influence of hydronephrosis on the removal by the kidneys of living bacteria injected intravenously was difficult to make. We studied transverse sections of the kidneys taken in the midportion in order to get fairly uniform sections which would include all elements of the renal structure. However, the bacterial colonies were not evenly distributed. Therefore we could not count bacterial colonies per field, but we estimated the number of colonies in the sections studied.

The bacterial colonies appeared as blue-staining masses whose shape was determined to some extent by the portions of the kidneys in which they were located. Colonies were seen in both normal and hydronephrotic kidneys, but were much more numerous in the latter. Their usual location was in the pelvic region, where they were commonly circular in section. It was, however, difficult to determine the exact anatomical location of these colonies because in growing they exerted a distorting force on surrounding structures and destroyed anatomical relationships.

In the cortex, colonies developed in the glomerular capillaries, where they grew as rounded colonies rather than in the crescentic shape that they would have to assume to fit between the glomerular tuft and the capsule.

Colonies were also seen interstitially in the medulla. Here, it was often difficult to determine whether they were located in tubules, blood vessels or lymphatics because the majority of them had assumed an elongated form running parallel to the tubules.

The results following injections of different bacteria were qualitatively similar. Those animals injected with *Bact. coli* showed slightly more colonies per section, although the location of the colonies was the same for *Bact. coli* and *B. typhosus*.

In an effort to approach more closely the conditions which might exist in clinical cases of ureteral occlusion, partial occlusion was produced in several rabbits by the method described. The results in these animals did not differ from those obtained after complete ureteral obstruction.

In several rabbits that had received intravenous injections of living bacteria, the liver and spleen also were examined by the in-

cubation and Gram staining procedure described. As might be expected, numerous colonies were demonstrated in both organs.

Discussion

It is now well known that localization of intravenously injected particulate matter occurs principally in those organs containing large numbers of phagocytic cells and a sinusoidal type of blood flow, whereas but little localization occurs in organs poorly supplied with macrophages and with a rapid flow of blood through vessels lined with common endothelium (Sullivan, Neckermann and Cannon [14]). For this reason, bacteria in the blood stream usually do not localize in the kidneys. It is not surprising, however, that at times larger particles, such as carbon, may lodge to some extent in the narrow lumen of the glomerular tuft or, if this is passed, in the smaller capillaries of the medulla.

In hydronephrotic kidneys, on the other hand, certain conditions tend to favor a localization of blood-borne particulate matter. Lucas [15] has shown that an increase in intrapelvic pressure slows the circulation of blood through the kidney and produces some renal damage, as evidenced by the finding of blood in the urine in the hydronephrotic sac. Helmholz and Field [16] have demonstrated that sterile inflammation occurs in the renal parenchyma and the pelvis after ligation of the ureter and that phagocytic cells become more numerous in the peripelvic areolar tissues and in the interstitial tissue of the involved kidney.

Other explanations have been advanced to account for the localization of blood-borne particulate matter in the kidneys. MacKenzie and Hawthorne [17] have demonstrated that carbon particles will remain in the renal substance for long periods after intravenous injection. Helmholz and Millikin [12] concluded that carbon particles may be found within the endothelial cells of the renal capillaries. Brickner [18] reported finding carbon particles within endothelial cells of the blood vessels after repeated heavy doses of carbon and attributed this to: (1) a "blockade" or saturation of the reticulo-endothelial system; (2) the tortuosity of the renal capillaries leading to a considerable stasis of blood flow; and (3) the smaller diameter of the capillaries furthering the approximation of particles to endothelium. We did not find carbon particles in endothelial cells of any of the material studied, pre-

sumably because the quantities of carbon which we used were too small to obtain this effect, and also because the endothelium of the blood vessels does not normally have any marked phagocytic activity.

From our data, and from a review of the literature, we feel certain that normal kidneys do not possess a marked tendency to localize or to excrete blood-borne bacteria or other particulate matter. When the ureter is occluded, however, or when overwhelming doses of particularly virulent bacteria reach the kidneys, pyelonephritis may occur. Clinically, there is frequently demonstrable obstruction of the ureters in cases of pyelonephritis, as has been pointed out by Lieberthal,[19] Weiss and Parker [20] and others. This finding has been borne out experimentally by Brewer,[21] Lepper,[22] and more recently by Mallory, Crane and Edwards.[23] Lepper has stressed the point that ureteral obstruction of even short duration may be sufficient to favor bacterial localization in the kidney. We feel that our findings confirm those of these authors. The sequence of events in the production of acute pyelonephritis has been well described by Mallory. We have shown how the bacteria lodge in these hydronephrotic kidneys as the first stage in the process leading to acute pyelonephritis.

Conclusions

1. The normal kidney usually does not excrete living bacteria circulating in the blood.

2. Blood-borne foreign particles have but a slight tendency to localize in the kidneys. Localization, when it occurs, is due to the narrow caliber of the glomerular and medullary capillaries, which tend to trap large or clumped particles.

3. Acute experimental hydronephrosis increases the tendency for blood-borne particulate matter to localize in the kidney.

4. This localization results from stasis, reinforced by the sterile inflammatory process and the mobilization of phagocytic cells.

Note: We wish to thank Paul R. Cannon for valuable assistance and advice during the course of this work.

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DESCRIPTION OF PLATE

PLATE 56

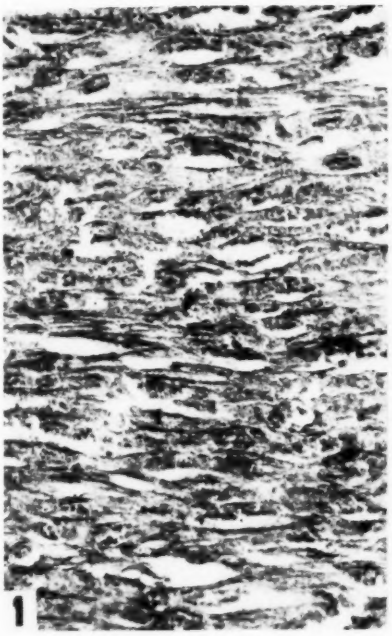
The kidneys from which these photomicrographs were made had been incubated for 24 hours before fixation, with resulting loss of cellular detail through autolysis.

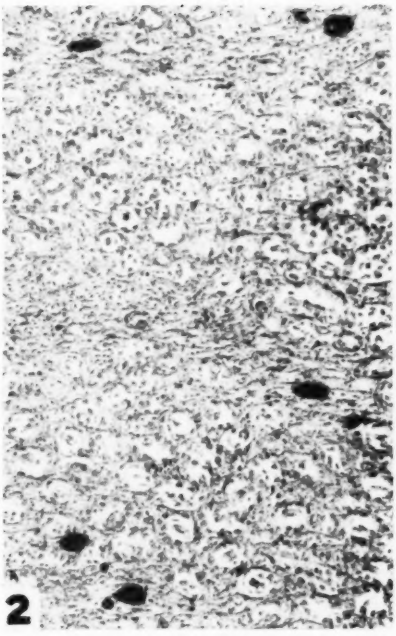
FIG. 1. Rabbit No. 9. This animal received 1 cc. of a 24-hour broth culture of *Staphylococcus aureus* intravenously and was sacrificed 2 hours later. This is a section of the normal kidney showing a few bacterial colonies which are quite small. They appear to be lodged between the tubules, probably in capillaries. × 125.

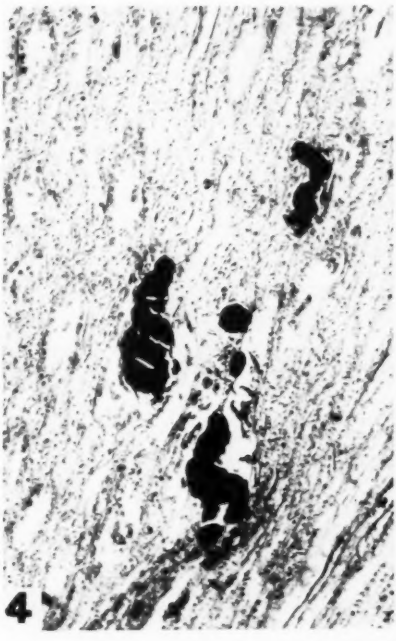
FIG. 2. Rabbit No. 9. Photomicrograph of a hydronephrotic kidney. The dark-staining areas are bacterial colonies. The elongated colonies seem to lie between the tubules, whereas the rounded ones are probably within blood vessels or are lying free in the tissue spaces. × 125.

FIG. 3. Rabbit No. 10. This animal was killed 4 hours after intravenous injection of 1 cc. of a broth culture of *Staphylococcus aureus*. Section of the normal kidney shows a few pin point bacterial colonies. × 125.

FIG. 4. Rabbit No. 10. A hydronephrotic kidney which contains several large bacterial colonies. One of these is surrounded by tubular epithelium. The others seem to lie in blood vessels. × 125.







Asher and Sokol

Bacterial Localization in Kidneys

UNIV OF MICH

EXOGENOUS TUMORS OF THE THYROID GLAND *

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The infrequency with which metastatic tumors are found in the thyroid gland long has attracted the attention of pathologists. This relative immunity to secondary implants, occurring in an organ whose blood supply per unit of weight exceeds that of any other in the body, has presented an interesting topic for speculation.

That this immunity may be more apparent than real has been suggested by a number of investigators, who have pointed out how seldom a careful study of the thyroid gland is made at routine postmortem examination. Willis [1] found metastatic lesions in the thyroid gland in 5.2 per cent of cases in which death was due to carcinoma. Rice [2] made painstaking microscopic studies of the thyroid glands in cases in which carcinoma was the cause of death and found embolic tumor cells in 8 of 89 glands examined.

Although the total number of cases with metastatic tumors of the thyroid gland which have been reported is comparatively small (less than 200), metastatic lesions have been described from tumors in almost every region of the body. There appears to be no correlation between the relative frequency of occurrence of various tumors and the incidence of metastatic lesions in the thyroid gland. Although the gastro-intestinal tract is the most common site of carcinoma, few cases have been reported in which tumors in this situation have spread to the thyroid gland. We were able to find only 3 reports in which the colon or rectum was the primary site of disease (Naegeli,[3] Willis,[1] and Rankin and Fortune [4]). Carcinoma of the breast, melanoblastoma and carcinoma of the lung have been most frequently described as establishing secondary foci in the thyroid gland. In most cases, metastatic lesions in the thyroid gland have been incidental findings at necropsy, but on a number of occasions thyroidectomy has dis-

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closed the presence of metastatic hypernephroma (Pemberton and Bennett,[5] H'Doubler [6] and Welti and Huguenin [7]).

Our interest in this condition was aroused by noting the development of a nodule in the right lobe of the thyroid gland of a patient who had undergone a combined abdominoperineal resection 6 months previously for adenocarcinoma of the rectum, grade I+. Extirpation of the right lobe of the thyroid gland revealed two hard, white, circumscribed nodules, which on histologic examination were found to resemble closely the rectal lesion. Accordingly, we have undertaken a study of all of the metastatic tumors of the thyroid gland which could be found in the museum of the Mayo Clinic.

Clinical Study

Nineteen cases were found in which metastasis to the thyroid gland had taken place. In 3 cases, thyroidectomy had been performed and led to a correct diagnosis during the life of the patient (2 of these cases have been reported by Pemberton and Bennett [5]). There were 7 cases in which some abnormality of the thyroid gland was noted on examination of the patient, but in which its true nature was not ascertained until after death. In 9 cases, metastatic lesions in the thyroid gland were an incidental finding at postmortem examination. In addition to these cases,

Table I
Distribution of Cases According to Origin of Primary Growth

Site of primary lesion	Confirmed cases*	Unconfirmed cases†
Lung	4	2
Kidney	2	1
Pancreas	2	0
Male breast	2	0
Female breast	0	4
Face	2	1
Stomach	1	0
Adrenal gland	1	0
Rectum	1	0
Cervix	1	0
Parotid gland	1	0
Bladder	1	0
Lymphosarcoma	1	0
Esophagus	0	6
Melanoblastoma	0	1
Sarcoma	0	2
Totals	19	17

* By pathologic study.
† No pathologic study made.

there were 17 cases in which there was clinical evidence of malignancy of the thyroid gland coexistent with or secondary to a neoplastic process elsewhere but in which no confirmatory pathologic study was carried out. There were 6 cases in which primary malignant lesions of the thyroid gland were coexistent with malignancy elsewhere.

The sites of the primary tumors in the group of 19 confirmed cases and 17 unconfirmed cases are shown in Table I. The remainder of this study is limited to a consideration of the confirmed cases.

In all but 2 cases, the metastatic lesions in the thyroid gland were part of a generalized carcinomatosis. In 1 of these, involvement of the regional lymph nodes was found at the time of operation on the primary lesion, but in the second no evidence of other metastatic lesions was found.

In 4 cases, the primary lesion had been removed surgically. In 2 cases, the examination of tissue removed by thyroidectomy was indicative of the presence of a primary lesion elsewhere. In 1 of these, the primary lesion subsequently was removed as there were no evidences of other metastatic foci.

In reviewing the cases it was found that in only one was there a history of previous disease of the thyroid gland. This patient, who had undergone thyroidectomy 12 years previously for exophthalmic goiter, returned with symptoms of recurrent hyperthyroidism and a basal metabolic rate of plus 27. The patient died from peritonitis following radium treatment of a squamous cell carcinoma of the cervix. At necropsy, the thyroid gland was found to contain metastatic carcinoma from the cervix. In 2 cases, "benign" goiter was described at the time of admission and in 4 cases, examination suggested "malignant" goiter, but in none of these cases was the ultimate pathologic diagnosis established during life. Among the 3 cases in which the diagnosis was made during life, there was a clinically "benign" goiter present in 2 and a clinically "malignant" goiter in 1.

Pathologic Study

In 7 cases, the implants of tumor tissue seemed to have been established in previously normal glands. In 1 of these an adenocarcinoma of the lung had gained entrance by direct invasion.

In 2 there was diffuse infiltration of the stroma of the gland by the cells of squamous cell carcinomas from the bladder and lip, respectively. In the remaining 4 cases the metastatic lesions were present as macroscopically circumscribed nodules, single in the case of a mixed tumor of the parotid gland and of an oat cell carcinoma of the lung and multiple in the case of neuroblastoma of an adrenal and of a hypernephroma. These circumscribed nodules may have represented invasion of preëxisting adenomas. In 12 cases, the involved glands were frankly adenomatous in varying degrees. In 1 of these cases the gland was invaded directly by a lymphosarcoma; in another indirectly by carcinoma of the lung which had extended into the thyroid gland from an involved supraclavicular lymph node. Diffuse infiltration of the thyroid gland occurred in the case of an adenocarcinoma of the stomach, grade IV, and of a squamous cell carcinoma of the cheek. In the former, the acini themselves were invaded to an unusual degree, in contrast to the more common limited invasion of the connective tissue elements of the gland with secondary displacement and destruction of the parenchyma. In the remaining 8 cases, macroscopically circumscribed metastatic nodules were present. In 1 of these, a tiny, solitary, metastatic lesion from a squamous cell carcinoma of the bladder was noted. In the remainder (1 from an adenocarcinoma of the rectum, 2 from adenocarcinomas of the pancreas, 2 from adenocarcinomas of male breasts, 1 from an adenocarcinoma of the lung and 1 from a hypernephroma), multiple metastatic nodules were present, either isolated or gathered around the walls of adenomas.

In a number of cases, the origin of the tumor cells could be surmised from a study of the thyroid gland alone. Hypernephroma and adenocarcinoma of the bowel could be identified from their metastatic lesions. In all cases, the grade of the metastatic tumor corresponded with the grade assigned to the primary tumor. There seemed to be little possibility of confusing any of the lesions studied with primary neoplasms of the thyroid gland. Varying degrees of fibrosis, necrosis and lymphocytic infiltration were noted.

Comment

It has long been recognized that primary carcinoma of the thyroid gland customarily arises in goitrous glands. It would also appear that metastatic tumors are found with greater frequency in adenomatous glands than in normal glands. This was true in 12 of the cases in this study and possibly in 4 others out of a total of 19.

Because of the infrequency with which the thyroid gland is involved by metastatic lesions, it has been suggested that the normal thyroid gland does not afford the "congenial soil" for tumor implants of which Paget [8] has written. Some have felt that the high oxygen content of thyroid tissue is a deterrent to the growth of malignant cells owing to their relatively anaerobic metabolism (Warburg [9]). The fact that exogenous neoplasms are more common in the adenomatous gland suggests that the structurally altered gland is more vulnerable to circulating tumor cells than the normal gland. It may be that the changed vascular condition favors the arrest of emboli, or that the abnormal thyroid tissue with its lower oxygen content offers a metabolically more suitable environment to the tumor cells. This phase of the subject has been thoughtfully considered and elucidated by Willis.[1] He has suggested also that other factors play a rôle. Since less common tumors such as melanoblastoma and lung tumors are relatively more common in the thyroid gland, it may be that their cells possess some special affinity for the thyroid gland.

Marked metabolic changes as a result of metastatic lesions in the thyroid gland have been described, ranging from myxedema (Willis [1]) to toxic or exophthalmic goiter (Mori,[10] H'Doubler,[6] Welti and Huguenin,[7] and Weiskittel [11]). In only 2 of our cases were there any symptoms suggestive of hyperthyroidism and in only 1 was there an elevation of the basal metabolic rate.

Metastatic lesions in the thyroid gland, although comparatively rare, probably occur more frequently than is generally suspected. Sudden changes in the size of the thyroid gland of a person known to be suffering from malignant disease elsewhere in the body, are suggestive of metastatic lesions in the thyroid gland. On rare occasions the removal of a goitrous gland may reveal the correct diagnosis in a patient suffering from a wholly unsuspected malignant process.

SUMMARY

A study is presented of 19 cases in which the thyroid gland was involved by a malignant tumor that arose elsewhere in the body. Metastatic tumors are found to occur more commonly in the adenomatous thyroid gland than in the normal thyroid gland.

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A MORDANT PREPARING FORMALDEHYDE-FIXED NEURAXIS TISSUE FOR PHOSPHOTUNGSTIC ACID HEMATOXYLIN STAINING *

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One of the most widely employed and consistently useful stains for the routine study of the neuraxis long has been Mallory's [1] phosphotungstic acid hematoxylin. This stain gives brilliant differential staining, familiar to all neuropathologists, on brain tissue fixed in Zenker's fluid. However, in most laboratories doing routine studies on human brains and cords it is inconvenient, if not technically undesirable, to use a number of fixatives. In this laboratory, fixation in a solution of formaldehyde before sectioning the brain has been used for some time, and mordants of various types have been used before staining.

Refixing the desired blocks of tissue in Zenker's solution has proven inadequate. For several years we have had quite satisfactory results from the phosphotungstic acid hematoxylin stain after formaldehyde fixation by following Kernohan's [2] suggestion and mordanting in Weigert's mordants I and II. This method consumes several added days in the preparation of the sections. In recent months the great increase in cost of fluorchrome ($CrF_3.4H_2O$) and the difficulty in obtaining it has made it desirable to find some other method. One of us (J.P.M.) has conducted a number of experiments with various chromium salts as mordants and the following method has been found eminently satisfactory.

PROCEDURE

1. Fixation in 4 per cent aqueous solution of formaldehyde. (Tissue stored in this fixing fluid for several years has been found to give good results.)
2. Cut blocks not over 5 mm. in thickness.
3. Wash blocks in running water 6 to 12 hours.

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4. Dehydrate with graded alcohols, 70 per cent, 80 per cent, 95 per cent, in the usual manner.
5. Complete dehydration of blocks with normal butyl alcohol, two changes of 4 hours each. (Any other routine method, such as absolute alcohol, xylol, acetone or dioxane [diethylene dioxide], is equally satisfactory.)
6. Embed in paraffin.
7. Cut sections, fix on slides, deparaffinize and hydrate to distilled water.
8. Place sections in *chromium chloride mordant* for 2 hours or longer. (Twenty-four and 48 hours in the mordant have caused no change in staining qualities.)
9. Rinse in distilled water.
10. Place sections in 0.25 per cent aqueous potassium permanganate solution for 10 to 15 minutes.
11. Rinse in distilled water.
12. Bleach in 5 per cent oxalic acid (until sections have lost brown tinge).
13. Rinse in distilled water.
14. Stain with Mallory's phosphotungstic acid hematoxylin 6 to 12 hours. (If Mallory's newer formula is used, potassium permanganate oxidation is necessary, as in his original formula.)
15. Decolorize in 95 per cent alcohol, two to three changes.
16. Dehydrate, clear and mount.

The *chromium chloride mordant* is prepared by dissolving 5 gm. of chromium chloride, green crystals ($CrCl_3.XH_2O$),* in 100 cc. of distilled water and adding 5 cc. of glacial acetic acid. This makes a dark green solution, which on standing takes on a dark purple-black color. This change is of no significance and the solution is usable after many weeks of standing. The cost is considerably less than one tenth the cost of fluorchrome.

The results from use of this mordant on formaldehyde-fixed material in our hands have been equally as brilliant as the results of phosphotungstic acid hematoxylin stains used on material fixed in Zenker's fluid. The differential staining of glial fibrils, fibrin, connective tissue, nuclei and cells is completely retained. Of

* Obtainable from the General Chemical Company, New York, N.Y.

course, numerous other stains may be used on sections from the same block since the mordant is applied to the sections and not the block.

Summary

A method is outlined for mordanting sections of neuraxis tissue, fixed in solutions of formaldehyde, to make them receptive to Mallory's phosphotungstic acid hematoxylin stain. The method is simple and lends itself to routine use. The mordant material—chromium chloride—is much more cheaply and easily obtained than fluorchrome.

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